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Design of responsive and degradable supramolecular host-guest polymer nanostructures for therapeutic applications

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Gutachter:

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Documentation of authorship

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Author	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
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P2	P. Wei,[1] S. Götz,[2] S. Schubert,[3] J. C. Brendel,[4] U. S. Schubert,[5] "Accelerating the acidic degradation of a novel thermoresponsive polymer by host-guest interaction", *Polym. Chem.* **2018**, *9*, 2634-2642.				
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Author	**1**	**2**	**3**	**4**	**5**	**6**	**7**
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P5	P. Wei,[1] J. A. Czaplewska,[2] L. M. Wang,[3] S. Schubert,[4] J. C. Brendel,[5] U. S. Schubert,[6] "Straightforward access to glycosylated, acid sensitive nanogels by host-guest interactions with sugar modified pillar[5]arenes", *ACS Macro Lett.* **2020**, *9*, 540-545.

Author	**1**	**2**	**3**	**4**	**5**	**6**
Conceptual development	x				x	
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Synthesis & characterization of monomers	x					
Nanogels synthesis & characterization	x					
SEM analyses			x			
Preparation of manuscript	x					
Correction of manuscript		x	x	x	x	x
Proposed publication equivalent	1.0					

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1. Introduction

Supramolecular polymers, unlike traditional covalent polymers, are composed of monomeric units which are held together by highly directional and reversible non-covalent interactions.[1] These interactions include hydrogen bonding, π-π interaction, metal coordination and host-guest interaction.[2] For the long-term usage, supramolecular polymers show several advantages: The reversibility of the non-covalent interaction in the supramolecular polymers facilitates modifications and rearrangement of the polymers. The dynamic interaction endows these polymers with unique abilities to react to external stimuli and adapt to significant environmental changes compared to conventional covalent polymers.[3] Stimulated by these advantages and the rapid growth of supramolecular chemistry, novel smart supramolecular polymer nanocarriers were among the most promising developments for controlled encapsulation and localized release of active pharmaceutical ingredients, including drugs, proteins and genes.[4] Among the different supramolecular interactions, the host-guest complexation by macrocycles has gained increasing attention in recent year, because this type of interaction can conveniently be used to bind two chemical entities together which, for example, enables to tune properties or functionalize materials. Up to now, a wide variety of macrocycles including crown ethers,[5] cucurbiturils,[6] cyclodextrins,[7] or pillar[5]arenes[8] have been synthesized and used for polymer modifications or supramolecular nanocarriers. All these materials feature a macrocyclic structure which comprise a modifiable exterior and a hydrophobic cavity (host) facilitating the selective inclusion of specific guest molecules. If the host and the guest are selectively modified with hydrophobic or hydrophilic polymers, respectively, amphiphilic quasi-block copolymers can be formed spontaneously by the resulting supramolecular interactions (Figure 1.1).[9, 10] The self-assembly of such supramolecular amphiphilic copolymers has been studied by various groups creating a range of nanostructure morphologies and tailor-made nanocarriers including micelles and vesicles which respond to different stimuli. In particular, cyclodextrin is often used for modification of hydrophilic polymers, while the corresponding guests are connected with hydrophobic polymers.[11-13] Based on the resulting host-guest interactions, quasi-block copolymers could be obtained, which can then self-assembly into nanocarriers for drug delivery.

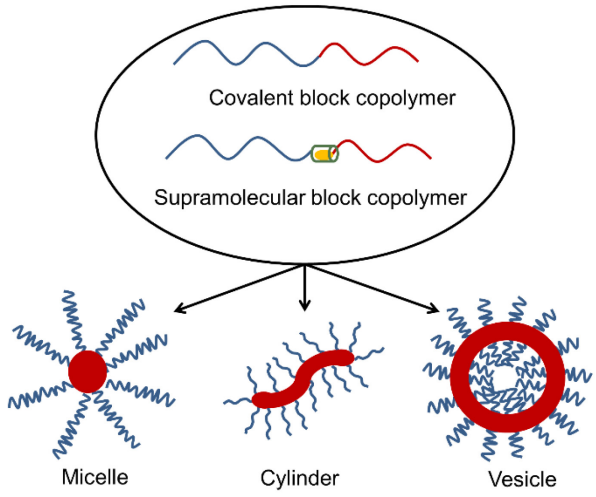

Figure 1.1 Schematic representation of covalent and supramolecular block copolymers and their self-assembly behavior.

In contrast to covalently bond amphiphilic polymers, the disassembly of these nanocarriers can be triggered by disturbing the host-guest interaction, which leads to the release of encapsulated compounds.[14] Besides the linear combination of two polymers by host-guest complex formation, these supramolecular interactions found widespread application in tuning the properties of functional polymers or to modify polymer based nanocarriers. In the former case, cyclodextrin for example can be used to tune the lower critical solution temperature (LCST) behavior of theromeresponsive polymers .[15-18] Supramolecular host-guest interactions were also applied to reversibly modify preformed polymer nanostructures. Either guest molecules or hosts, such as cyclodextrin, were incorporated into then nanostructures which enable the subsequent addition of functional groups (targeting units, dyes, cationic groups) attached to the complementary group. As a result, nanostructures can be functionalized for targeted delivery, selective release, imaging, or gene transfection.[19]

The pillar[5]arenes as a new generation of supramolecular compounds have attracted increasing interest due to the ease of modifying their exterior groups, as well as the wide range of suitable guests that form strong inclusion complexes.[20-22] In contrast to well-studied cyclodextrins, however, the potential of these pillar[5]arenes has not been fully exploited, yet. The overall aim of this thesis was therefore to investigate the capacities of pillar[n]arene based materials in polymer chemistry which includes (1) the formation of supramolecular quasi-block copolymers by host-guest interactions and their formulation into nanoparticles, (2) the tuning of thermoresponsive polymers, (3) as well as using pillar[5]arene to functionalize preformed nanoparticles or nanogels, respectively, for therapeutic applications.

In more detail, the first chapter of this thesis (Chapter 2.1) deals with the formation of quasi-amphiphilic copolymers based on the host-guest interaction between pillar[5]arene modified hydrophilic polymers and viologen modified polycaprolactone (PCL). Subsequently, the materials were investigated for their ability to self-assemble in water and for their response to pH changes as well as their effect on enzymatic degradation. In parallel to this work, the influence of such host-guest interactions on degradable and thermoresponsive polymers was studied using different hydrophilic pillar[5]arenes (Chapter 2.2). In this part, a new thermoresponsive polymer poly(*N*-[(2,2-dimethyl-1,3-dioxolane)methyl]acrylamide) (PDMDOMA) was further introduced, which features a similar LCST behavior as poly(*N*-isopropylacrylamide) (PNIPAM),[23, 24] poly(*N*-vinylcaprolactam) (PVCL)[25, 26] or poly(oligo(ethylene glycol) acrylate) (POEGA)[27] (Figure 1.2), but in addition comprises a labile acetal group, which can degrade under acidic conditions.[28-30]

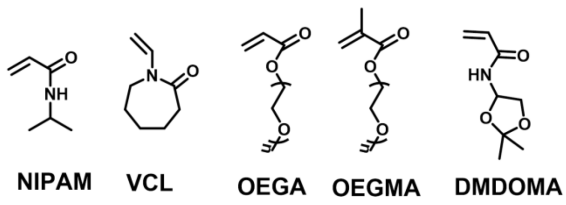

Figure 1.2 Schematic representation of monomers used in the preparation of thermoresponsive polymers.

Based on the experience from these studies, the work on these thermoresponsive and degradable polymers was expanded in Chapter 3. Taking advantage of the well-established procedure for preparation of nanogels by

precipitation polymerization, the monomer DMDOMA was first tested to be suitable for these techniques and a series of nanogels with three different crosslinkers and acrylic acid as comonomer was prepared. In contrast to the commonly used monomers NiPAM,[31-33] OEGA[34, 35] or VCL,[36-38] the hydrolyzable acetal groups renders the nanogels sensitive to acidic environments. Consequently, these novel nanogels were investigated for their ability to encapsulate hydrophobic drugs and selectively release them at acidic conditions as they are common in endo/lysosomal pathways.[34, 39] In order to broaden the scope of these structures, the experiences from Chapter 2 were utilized to further modify and functionalize these nanogels. One limitation of these nanogels is the constant content of acrylic acid (10% in total) which is crucial for stability. As the content of carboxylic acid groups was found to determine the hydrolysis rate (Chapter 2.2), host-guest interactions were investigated in Chapter 4 to tune this content by post polymerization reactions. For this purpose, a pyridinium salt as an alternative monomer to acrylic acid was combined with DMDOMA to first create nanogels stabilized by a positive charge. The pyridinium is a well-studied guest for carboxylate-pillar[5]arene allowing to conveniently tune the content of carboxylic acid in the nanogels by addition of various amounts of carboxylate-pillar[5]arene. In addition to the tuning of the hydrolysis rate, this successful integration of a guest moiety into the nanogel opens up a straightforward pathway for the introduction of targeting ligands on the surface of the nanogels. In the second part of Chapter 4, this approach was used to introduce sugar modified pillar[5]arenes to the surface of the nanogels, which are known to selectively interact with lectins, *i.e.* proteins on the membranes of cells, which are often overexpressed in cancer.[40] To verify the selectivity of the modified nanogels, the interaction with Concanavalin A (Con A) was tested, as this lectin specifically binds to α-D-mannosyl and α-D-glucosyl residues.[41]

A detailed overview of the work is given in the following chapters, and the final results and conclusions are subsequently summarized in Chapter 5 (German: Chapter 6).

2. Supramolecular polymers: Formulation, self-assembly and responsiveness

Parts of this chapter have been published in: **P1**) P. Wei, F. H. Sobotta, C. Kellner, D. Bandelli, S. Höppener, S. Schubert, J. C. Brendel, U. S. Schubert, *Polym. Chem.* **2020**, *11*, 1985-1997. **P2**) P. Wei, S. Götz, S. Schubert, J. C. Brendel, U. S. Schubert, *Polym. Chem.* **2018**, *9*, 2634-2642.

Supramolecular chemistry has been intensively utilized in the fabrication and modification of copolymers as an alternative to common covalent methods. In this chapter, pillar[5]arene derivatives, viologen salts and pyridinium salts as the common host and guest combination are used with series of hydrophilic polymers (PNAM, PNAMP), hydrophobic polymers (PCL, viologen-PCL) and the thermoresponsive polymer (PDODOMA) to study their behavior, including the host-guest interaction, self-assembly, influence on their degradation, hydrolysis and thermoresponsiveness.

2.1 The influence of supramolecular host-guest interaction with pillar[5]arene and viologen on the construction of quasi-block polymer nanoparticles

In the past decades, block copolymers have become versatile and widely used materials for the formation of nanostructures that rely on the self-assembly of amphiphilic polymers forming different morphologies such as rods, micelles, and vesicles.[42, 43] Despite the success of this work in certain aspects, it still suffers from limitations due to the complicated and time-consuming synthesis of amphiphilic copolymers with the right hydrophobic-hydrophilic ratio that is required to gain the desired morphologies.[44] To overcome these problems and to broaden the family of nanostructures based on amphiphilic polymer, a variety of novel quasi-block copolymers were prepared with pillar[5]arene modified hydrophilic poly(*N*-acryloyl morpholine) (PNAM-P[5]) or pH-responsive poly(*N*-acryloyl-*N'*-methyl piperazine) (PNAMP-P[5]) and the viologen modified hydrophobic polycaprolactone (Viologen-PCL). Their ability to form host-guest interactions was investigated and the resulting supramolecular amphiphilic polymers tested for self-assembly in water and enzymatic degradation.

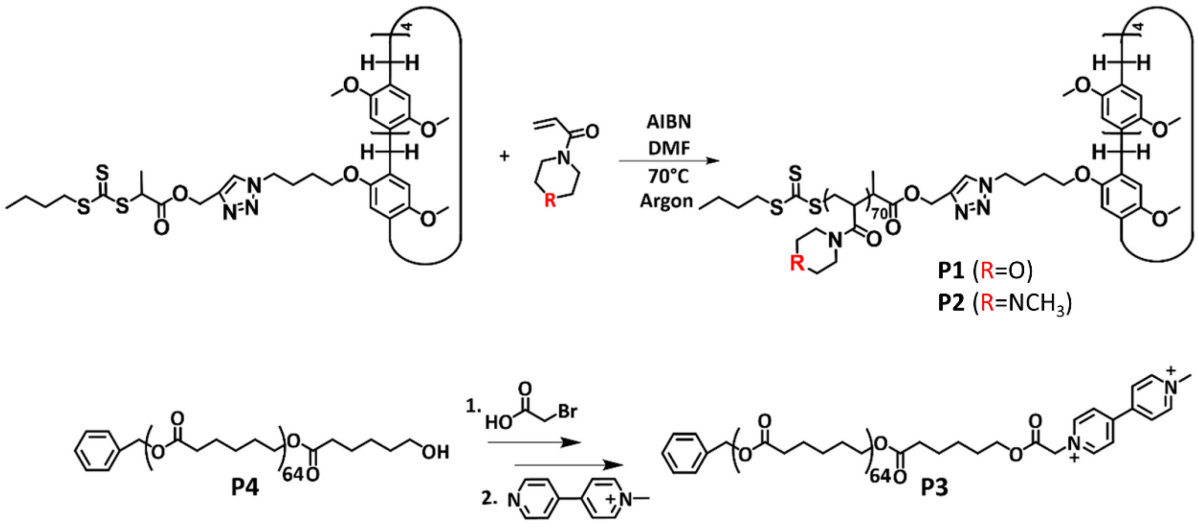

Figure 2.1 Schematic representation of synthesis routes to polymers **P1** to **P4**.

In order to successfully fabricate the supramolecular copolymers, the most critical step is the modification of a polymer with the corresponding host or guest. For this purpose, pillar[5]arene was first connected to the chain transfer agent (CTA) with an alkyne modified trithiocarbonate in a copper-catalyzed azide-alkyne cycloaddition (CuAAC).[45] Subsequently, the pillar[5]arene modified hydrophilic polymers poly(*N*-acryloyl morpholine)-pillar[5] (PNAM-P[5], **P1**) and poly(*N*-acryloyl-*N*'-methyl piperazine)-pillar[5]arene (PNAMP-P[5], **P2**) were both synthesized by reversible addition-fragmentation chain transfer (RAFT) polymerization with azodiisobutyronitrile (AIBN) as the initiator (Figure 2.1).[46] The polycaprolactone (PCL) polymer **P4** and viologen modified PCL (**P3**) were synthesized according to reported procedures (Figure 2.1).[47] All the polymers were characterized by ^{1}H NMR spectroscopy to determine their molar mass and further confirm the successful end group modification of **P1**, **P2** and **P3** by observation of all the representative signals of either the pillar[5]arene or the viologen units in the spectra after purification. Additionally, size exclusion chromatography (SEC) studies revealed their narrow distributions (D<1.2) (Table 2.1).

Table 2.1 Overview of selected characteristics of the polymers.

Abbrev.	DP (NMR) [a]	M_n (NMR) (g mol^{-1})	M_n (SEC)[d] (g mol^{-1})	M_w (SEC) (g mol^{-1})	*Đ*
P1	70 [b]	11,000	9,600	10,600	1.10
P2	70 [b]	11,900	9,600	11,500	1.19
P3	64 [c]	7,700	10,700	11,200	1.04
P4	64 [c]	7,400	10,700	11,100	1.04

[a] Determined from ^{1}H NMR analysis. [b] Calculated from the signal intensity of the proton of the pillar[5]arene (δ = 6.5 ppm) in comparison to the proton signal of the hexa-atomic ring of the polymers (δ = 3.5 ppm). [c] Calculated from the signal intensity of the proton on the benzene ring (δ = 7.4 ppm) in comparison to the proton signal of the methylene connected with the acyl group of polymers (δ = 4.1 ppm). These ratios were used to calculate the DP. [d] SEC: DMAc + 0.21 wt.% LiCl, poly(methyl methacrylate) (PMMA) calibration.

The complexation of the respective polymer components is crucial for the successful preparation of the quasi-block copolymers. ^{1}H NMR spectroscopy was used as the general method to confirm the complexation.[48] In the corresponding spectrum, the peaks belonging to the guest **P3** disappear due to their inclusion in the cavity of pillar[5]arene in **P2** (Figure 2.2). Surprisingly, the same phenomenon could not be observed in the complexation of **P1** with **P3**, which is most likely related to a weaker complexation ability between them. In order to analyze their complexation strength, titration experiments were performed to see changes of the fluorescence intensity of the pillar[5]arene.[48] These results revealed that the association constant (K_a) of **P2**+**P3** is (9.3±0.49)×10^4 M^{-1} and is stronger than the K_a of **P1**+**P3** ((3.55±0.22)×10^4 M^{-1}), which confirms the previous assumption.

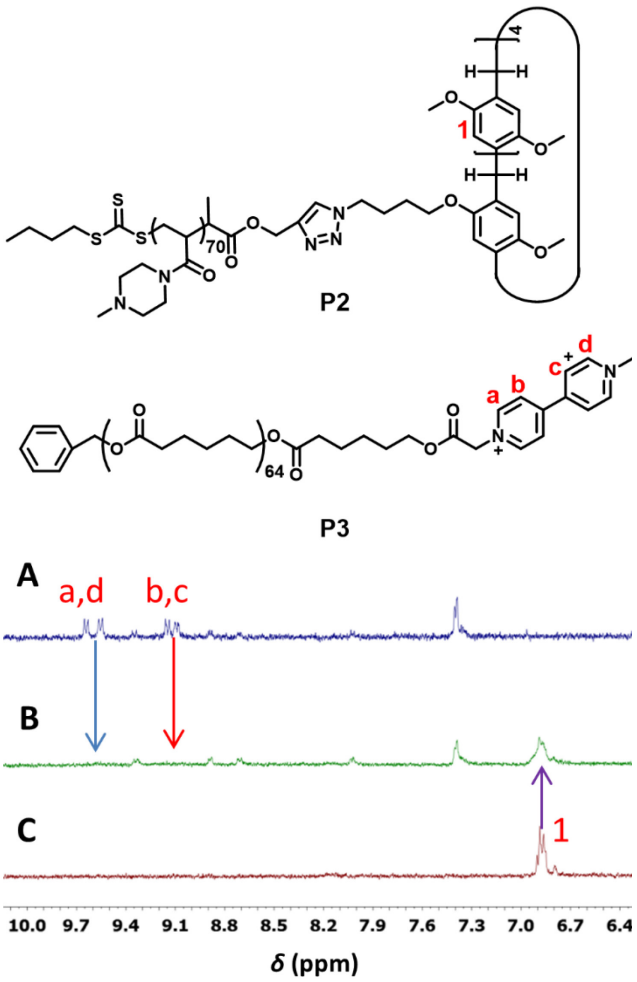

Figure 2.2 ^{1}H NMR (acetone-d_6, 300 MHz, 25 °C) spectra of A) 4 mM **P3**, B) complex of **P3** (4 mM) with 1 eq. **P2**, C) **P2** (4 mM).

Nanoparticles were prepared by nanoprecipitation dropping the solution of the quasi-block copolymer in acetone into water. This method was chosen due to its excellent reproducibility without tedious processing steps.[49, 50] As listed in Table 2.2, stable nano-sized micelles were obtained in pure aqueous solution for the neutral polymer **P1** as well as the pH-responsive polymer **P2** in combination with **P3** (viologen-PCL). The stability of the formed micelles in buffered saline solutions was further tested to evaluate their potential application in biological fields. It is noteworthy that the micelles formed with **P3** (viologen-PCL) and neutral **P1** (PNAM) aggregated quickly in less than one day.

Initially thought as control experiments, further nanostructures were prepared by using **P3**, **P4** and **P2**+**P4** under the same conditions, respectively. In pure water, all of them formed nanoparticles, whereas the formulation of **P2**+**P4** was even stable in buffer solutions, which is surprising when considering the strong tendency of all particles to aggregate in buffer even when forming host-guest complexes with **P1** and **P3**. One assumption is that the strong hydrophobicity of pillar[5]arene in **P2** is incorporated into the hydrophobic core formed by **P4**, while the slightly charged hydrophilic part in combination with pillar[5]arene is able to stabilize the nanoparticles. Further studies on the morphology of **P2**+**P3** and **P2**+**P4** were done by transmission electron

microscopy (TEM) or *cryo*-TEM and revealed that the size of the micelles based on **P2**+**P3** is 20 to 70 nm, while nanoparticles formed from **P2**+**P4** showed sizes ranging from 50 to 200 nm (Figure 2.3).

Table 2.2 Summary of the characteristics of the different nanostructures.

Abbrev.	Z-Ave (nm)	PDI	Z-Ave (nm)	PDI
P1+P3	33	0.34	Aggregation	
P2+P3	43	0.23	41	0.13
P2+P4	90	0.21	85	0.15
P3	44	0.36	Aggregation	
P4	64	0.15	Aggregation	

[a] Nanostructures were measured in H_2O at 25 °C. [b] Nanostructures were measured in phosphate buffer (10 mM, pH 7.4) at 25 °C.

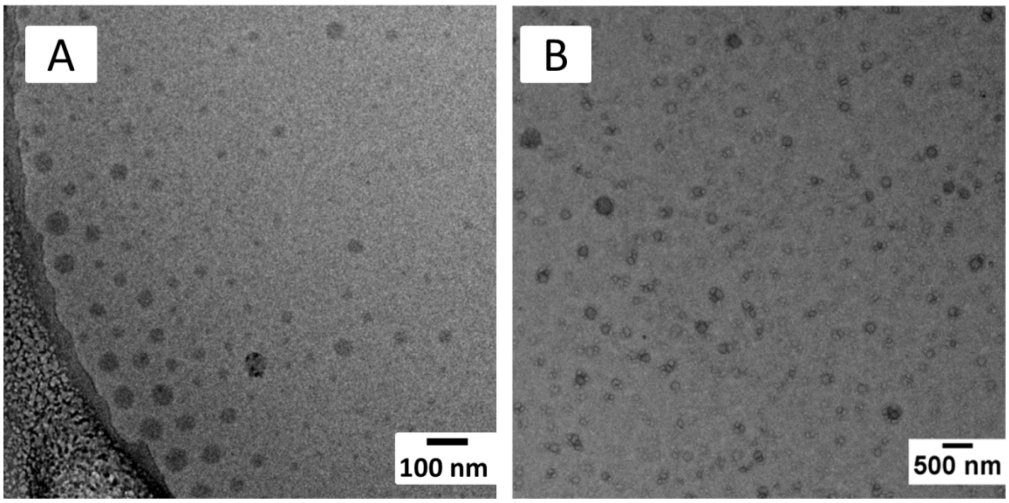

Figure 2.3 A) *cryo*-TEM image of **P2**+**P3** and B) TEM image of **P2**+**P4**.

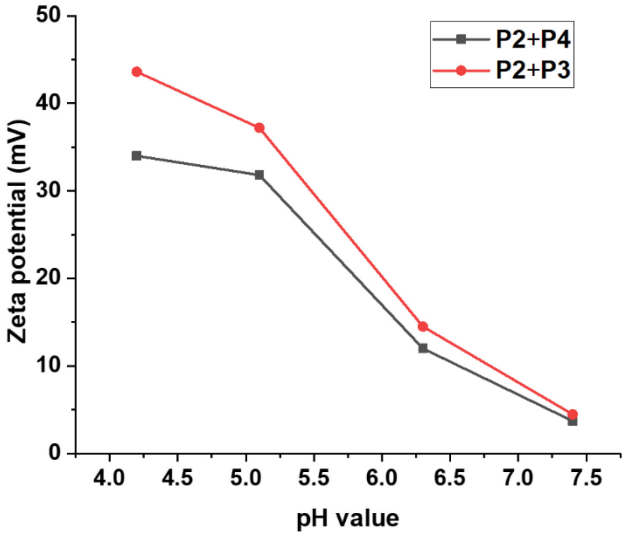

Figure 2.4 Zeta potential of A) **P2**+**P4** and B) **P2**+**P3** at different pH values measured by DLS.

Due to the fact that the acid dissociation constant (pK_a) of tertiary amino groups in polymer **P2** is 5.2 as estimated by titration experiments, almost all of these tertiary amino groups can be deprotonated at a physiological pH value of 7.4. Thus, the corresponding micelles formed by **P2** as shell might follow a similar trend. As a consequence, both micelles prepared from **P2** (**P2**+**P3** and **P2**+**P4**) were examined for their pH-responsive behavior in the relevant pH values ranging from 4 to 7.4. The zeta potential of both micelles increases with decreasing pH value (Figure 2.4). Considering the increasing charge repulsion produced from the protonation of **P2** with decreasing pH value, it might cause the dissociation of the complex of **P2**+**P3** or a detachment of the stabilizing shell in the combination of **P2** and **P4**. The size, distribution and stability of both micelles were estimated by dynamic light scattering (DLS) at various pH values. Only the micelles of **P2**+**P4** showed a reversible behavior when the pH was decreased from 7.9 to 3.1 and then reversed to 7.9 again, while the micelles of **P2**+**P3** resulted in irreversible aggregates in the same conditions.

Considering their potential application as future nanocarriers for controlled release, the cytotoxicity of the particles was tested and the enzymolysis of PCL was examined at neutral and acidic conditions as it would appear in the endo-/lysosomal uptake pathway.[51, 52] Both micelles were stable with 1 U mL^{-1} lipase under physiological condition after incubation for two days (Figure 2.5 A), while a good biocompatibility was verified for the micelles as well as the pure polymer **P2** (Figure 2.5 B). However, micelles formed by **P2**+**P4** degraded much faster than the other one at acidic conditions. The reasons could be the different enzymatic activity at different pH values and the more pronounced exposure of PCL to the enzyme in acidic conditions, in particular for **P2**+**P4** due to the release of **P2** promoted by the repulsive forces.[53] Interestingly, the system offers the possibility of tuning the degradation rate. When using a mixture of the viologen units (0.5 eq. **P4** and 0.5 eq. **P3** plus **P2**), the resulting nanoparticles exhibited a moderate degradation rate in comparison to the above degradation results.

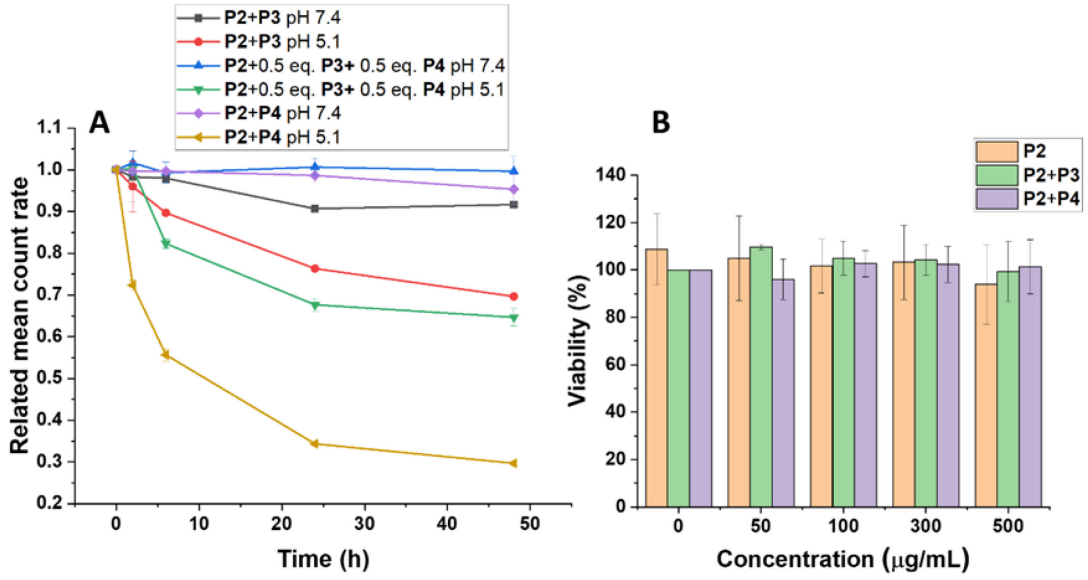

Figure 2.5 A) Count rate measurements (DLS) to monitor the enzymatic degradation (1 U mL^{-1} at different pH values. B) PrestoBlue assay to evaluate the cytotoxicity of the nanoparticles that were prepared from **P2**, **P2**+**P3** and **P2**+**P4** respectively using cell line L929.

Overall, the host-guest interactions between pillar[5]arene and viologen prove their potential for construction of quasi-block copolymers, which even tolerate the self-assembly into spherical nanostructures Moreover, the versatility of the host-guest interactions provided an excellent opportunity to selectively tune the resulting properties of the nanoparticles, such as the degradation rate in the presence of enzymes.

2.2 Influence of supramolecular structures on the LCST and hydrolysis behavior of PDMDOMA

In addition to the self-assembly of amphiphilic block copolymers, thermoresponsive polymers represent another interesting class of polymers, which are known for the ability to reversibly form aggregates in solution. These polymers can for example be soluble below the lower critical solution temperature (LCST), but will exhibit a volume phase transition above the LCST.[54, 55] This behavior is commonly related to the hydrogen bonding between the repeating units of polymers themselves and the water molecules in solution.[56, 57] Hydrophobic or hydrophilic components can commonly be incorporated by covalent or non-covalent bonds in order to tune their LCST behavior for adapting to various applications.[58-61] In the latter case, supramolecular host-guest interaction gain increasing attention in recent times as they offer a convenient way for modifications.[61, 62] PDMDOMA is a new monomer introduced only recently but features a similar LCST behavior as PNIPAM.[30] However, the additional pH-sensitive dioxolane group endows it more chances for applications in biological fields.[29, 63] It is well-known that the LCST of PNIPAM can be tuned using supramolecular host-guest interactions, which is in fact more convenient compared to the traditional covalent incorporation of comonomers.[61, 64] Similar to the complex formation between pillar[5]arenes and viologen salts in Chapter 2.1, here, different types of pillar[5]arenes modified with different hydrophilic moieties were used to study their influence on PDMDOMA, including the LCST and hydrolysis behavior.

Two well-studied water-soluble supramolecular hosts, carboxylate-pillar[5]arene (H1) and oligoethylene oxide-pillar[5]arene (**H2**), were chosen in this work.[65, 66] Pyridinium salts, which represent suitable guest moieties, were first connected to the chain transfer agent (CTA) and subsequently used in RAFT polymerization (Figure 2.6). The resulting polymers (**P5**, **P6**, **P7**) were analyzed by NMR and SEC and show narrow molar mass distributions (Table 2.3). ^{1}H NMR spectroscopy was used to prove the successful complexation between polymers and pillar[5]arenes.

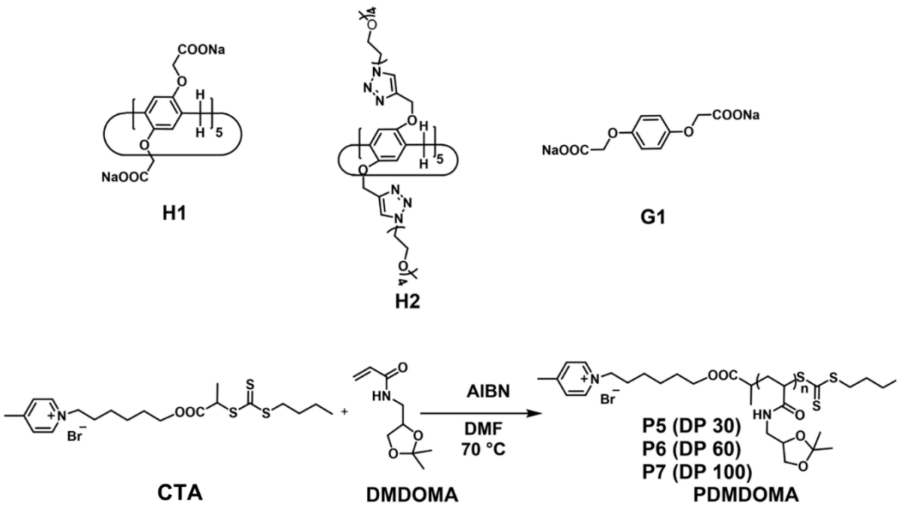

Figure 2.6 Schematic representation of **H1**, **H2**, **G1**, **CTA** and the synthesis route to **P5**, **P6** and **P7**.

Table 2.3 Overview of the characteristics of the PDMDOMAs **P5**, **P6** and **P7**.

Abbrev.	DP (NMR)	M_n (NMR)[a] (g mol^{-1})	M_n (SEC)[b] (g mol^{-1})	M_w (SEC)[b] (g mol^{-1})	*Đ*
P5	30	6,000	10,900	11,900	1.09
P6	60	11,600	17,500	20,000	1.14
P7	100	19,000	24,900	30,000	1.19

[a] Determined by ^{1}H NMR analysis (calculated from the signal intensity of the proton at the pyridine (δ = 8.92 ppm) in comparison to the proton peak integral of the hemiacetal (δ = 3.63 ppm) before post-modification). These ratios were used to calculate the DP. [b] SEC: DMAc + 0.21 wt.% LiCl, poly(methyl methacrylate) (PMMA) calibration.

The LCST behavior of thermoresponsive polymers can be influenced by their concentration and incorporation of hydrophilic segments.[67, 68] Temperature-dependent turbidity measurements were performed at different concentrations with or without 1 eq. of the two different pillar[5]arenes **H1** and **H2** in pure water (Figure 2.7). All the results revealed that the LCST shifts to higher temperature with the decreased concentration as well as with the complexation of pillar[5]arene. It is noteworthy that there was a clear difference between **H1** and **H2** in their influence on the LCST behavior. The reason might be the different hydrophilicity of the pillar[5]arenes themselves or the stronger complexation in the case of **H1** in comparison to **H2**. Therefore, their complexation behavior was investigated by isothermal titration calorimetry (ITC) to determine any differences in binding strengths using **P6** as an example polymer. The results displayed that the association constant between **P6** and **H1** (4.529×10^4 M^{-1}) is almost ten times higher than the association constant between **P6** and **H2** (7.223×10^3 M^{-1}). This difference might be explained by the additional electrostatic interaction between the negative charge on **H1** and the positive charge of the pyridinium salt on **P6**.

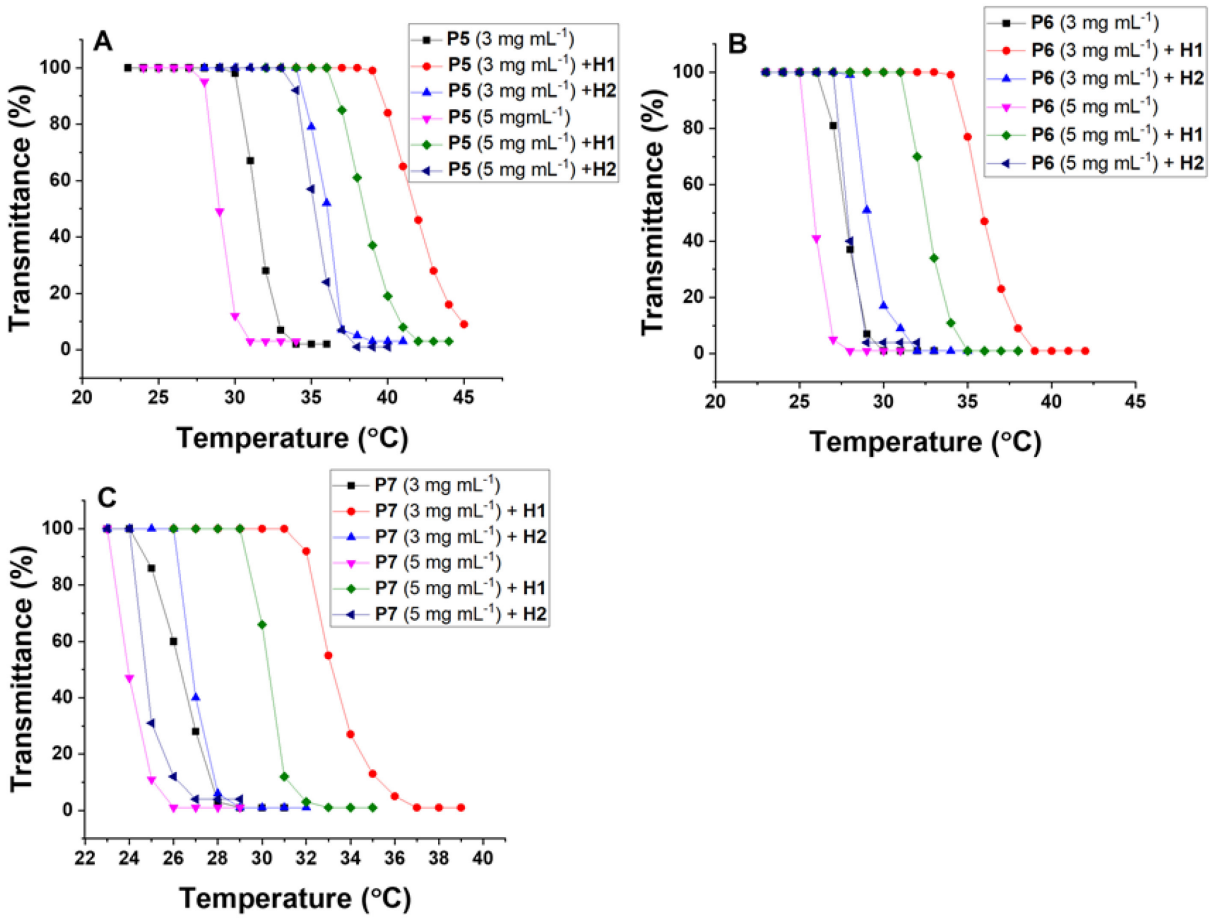

Figure 2.7 Transmittance changes of polymers (**P5**, **P6**, **P7**) with or without **H1** and **H2**. A) **P5** with and without 1 eq. **H1** or **H2**, B) **P6** with and without 1 eq. **H1** or **H2**, C) **P7** with and without 1 eq. **H1** or **H2**. Heating rate: 0.2 °C min^{-1}.

The hydrolysis behavior in PDMDOMA complexed with **H1** or **H2** were studied by NMR spectroscopy. Previous work proved already that carboxylate-pillar[5]arene (**H1**) can catalyze the hydrolysis of pH-sensitive bonds.[69] Similar to these studies, **P6** was hydrolyzed in acidic conditions releasing acetone which can be utilized as an excellent indicator to monitor this reaction in a closed environment (Figure 2.8). Therefore, time dependent ^{1}H-NMR measurements for all the samples with or without pillar[5]arenes were performed in acidic buffer solutions (pH = 5.2) incubated at 37 °C. At each time point, the signal for acetone was compared to the present acetate signal, which was used as an internal standard. Within the tested 11 days, a considerably faster hydrolysis was observed for the combination of polymers with H1 in comparison with the pure polymers (Figure 2.8).

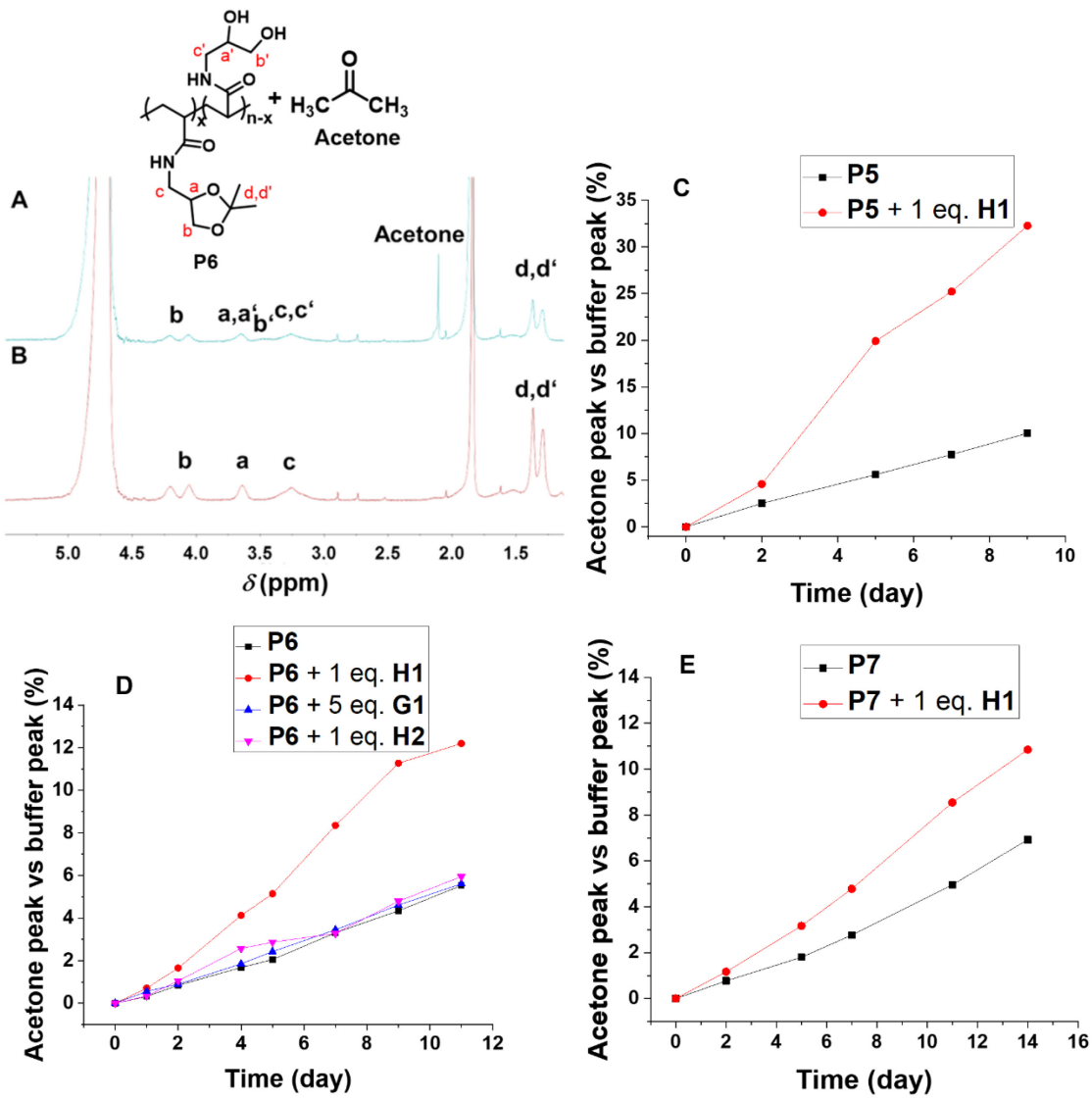

Figure 2.8 ^{1}H NMR spectra showing the hydrolysis of polymer **P6** (DP 60) at pH 5.2 at 37 °C: A) 5 mg mL^{-1} polymer **P6** after 11 days, B) 5 mg mL^{-1} polymer **P6** at 0 h. The relative hydrolysis of the polymers (5 mg mL^{-1}) at pH 5.2 C) **P5** with or without 1 eq. **H1**, D) **P6** with or without 1 eq. **H1**, 1 eq. **H2** and 5 eq. **G1** as well as E) **P7** with or without 1 eq. **H1**. The acetate peak in the ^{1}H NMR was used as an internal standard for comparison.

For comparison, **H2** and **G1** (subunit of **H1**) were tested under similar condition to investigate if the carboxylated groups of **H1** or also the cavity of **H1** play an important role in the hydrolysis of the polymer. While the hydrolysis rates of pure polymer **P6**, **P6** with 1 eq. **H2**, and **P6** with 5 eq. **G1** were all very similar, **P6** with **H1** is considerably faster than others An explanation might be that that polymer **P6** became better soluble in water with complexation of **H1** than the pure polymer itself, as the transition temperature was closer to the examined 37 °C. Furthermore, more water, which is essential for the hydrolysis of the acid sensitive group, was bound to the modified polymer by hydrogen bonding with the partially charged carboxylate groups of **H1**. Consequently, further hydroxyl groups would be unveiled after hydrolysis of the acetal groups, which subsequently coordinate

to additional water molecules accelerating the hydrolysis.[68] Based on these results, it can be said that carboxylate-pillar[5]arene did not only change the LCST of PDMDOMA but has a major influence on their degradation speed (Figure 2.8).

The results demonstrate that PDMDOMA not only has great potential for application in the preparation of multiresponsive nanocarriers but also allows the selective tuning of its properties by supramolecular interactions.

3. Functional nanogels by precipitation polymerization for encapsulation and controlled release of doxorubicin

Parts of this chapter have been published in: **P3)** P. Wei, G. Gangapurwala, D. Pretzel, M. N. Leiske, L. M. Wang, S. Hoeppener, S. Schubert, J. C. Brendel, and U. S. Schubert, *Biomacromolecules* **2019**, *20*, 130-140.

The results presented in Chapter 2.2 already prove the potential of the new monomer DMDOMA, however, a challenge remains the formation of well-defined nanostructures. An interesting approach used for such thermoresponsive polymers represents the precipitation polymerization to form crosslinked nanogels. It is well-known that such nanogels show advantageous properties such as high water content, large surface area for easy bioconjugation, high loading capacity, and long circulation in blood.[70] Thus, thermoresponsive behavior in *N*-isopropylacrylamide (NiPAM),[31-33] oligo(ethylene glycol) acrylate (OEGA)[34, 35] and *N*-vinylcaprolactam (VCL)[36-38] have been widely used to prepare nanogels using precipitation polymerization and have already been well described in a number of applications involving controlled drug encapsulation and targeted release. The precipitation polymerization is a common technique to fabricate nanogels that relies on the reversible phase transition of thermoresponsive polymers in aqueous solution at varying temperature due to the high conversion, incorporated a wide range of comonomers and convenient purification with dialysis or centrifugation.[34, 71, 72] Drugs can for example be encapsulated into the network by electrostatic interactions or Van der Waals forces between the hydrophobic drugs and the hydrophobic domains in the nanogels. So far, the release behavior mainly relies on the degradation of the crosslinkers with, *e.g.*, acetal or disulfide functionalities, which respond to differences in pH values or a reducing environment, which is, for the example given by higher GSH levels within the cytosol.[36, 73]

Inspired by the interesting thermoresponsive properties and the pH-sensitivity of PDMDOMA studied in Chapter 2.2, a variety of novel nanogels were proposed by using the DMDOMA with acrylic acid as co-monomer and three types of crosslinkers to prepare a series of nanogels. Apart from a potential release by degradation in crosslinkers, the hydrolyzable acetal groups should further facilitate an accelerated release under acidic conditions.

In order to obtain a new type of multi-responsive nanogels, three different crosslinkers including *N*,*N'*-methylene bisacrylamide (BIS), *N*,*N'*-bis(acryloyl)cystamine (BAC) and (propane-2,2-diylbis(oxy))bis(ethane-2,1-diyl)diacrylate (KTDA) were chosen in combination with DMDOMA. Acrylic acid was integrated as comonomer to stabilize the resulting nanogels and to avoid the use of additional surfactants, which are difficult to remove in purification.[74] All nanogels were prepared by precipitation polymerization in aqueous medium with KPS as the initiator (Figure 3.1). Notably, NaOH was used to maintain a basic pH value (pH 10) which prevents the hydrolysis of DMDOMA and deprotonates the acrylic acid. The resulting negatively charged acrylic acid (AA) can stabilize the nanogels and inhibit their aggregation due to electrostatic repulsion. All nanogels were analyzed by DLS in phosphate buffer, as well as by SEM in the dry state (Table 3.1, Figure 3.2).

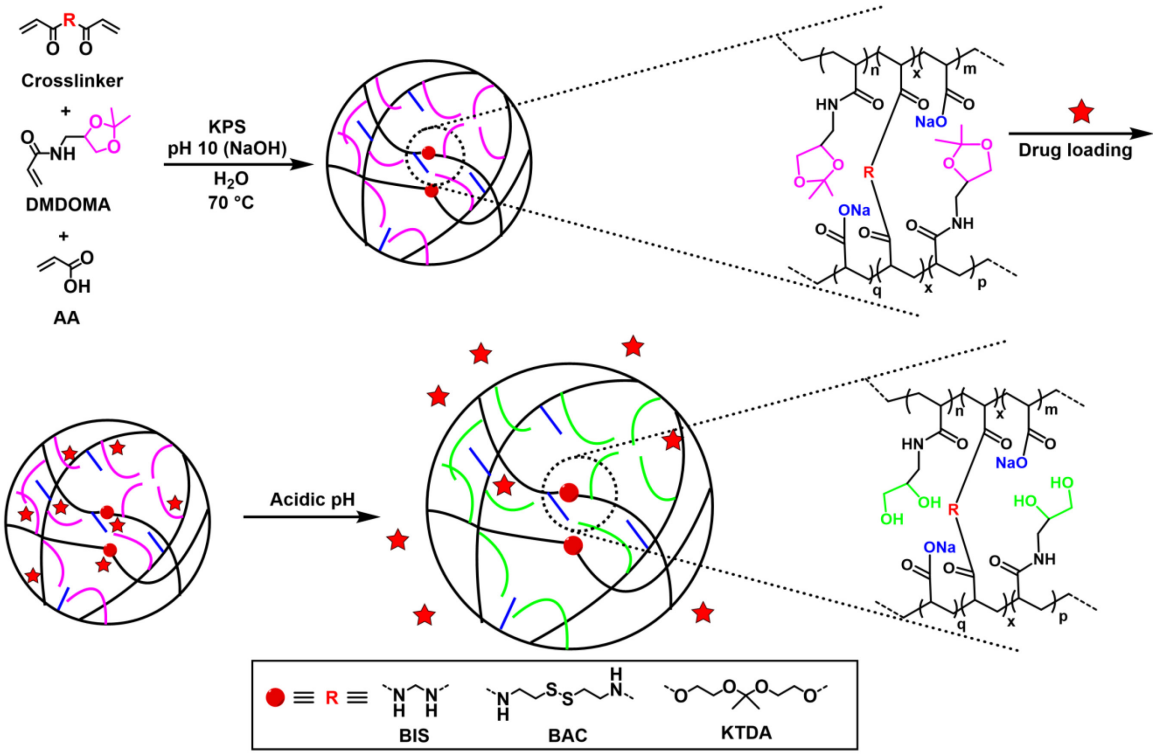

Figure 3.1 Illustration of the nanogel preparation, drug loading, and acid-triggered drug release from the nanogels.

Table 3.1 Characterization of nanogels by DLS measurements.

Sample	Z-Average (nm)	PDI[a]
PDA-BIS	150	0.217
PDA-BAC	97	0.093
PDA-KTDA	202	0.159

[a] Size was measured in phosphate buffer (10 mM, pH 7.4) at 25 °C.

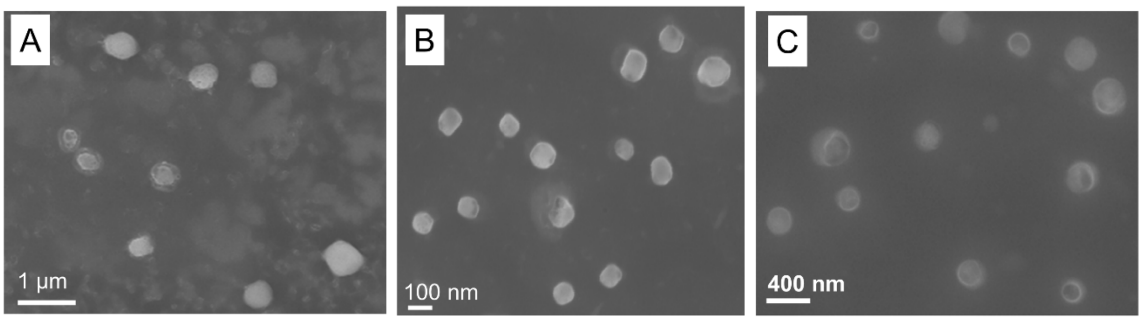

Figure 3.2 SEM image of A) PDA-BIS, B) PDA-BAC and C) PDA-KTDA nanogels.

As the nanogels were designed to facilitate the hydrolysis of the acetal groups under acidic conditions, ^{1}H NMR spectroscopy was used to monitor the release of acetone (hydrolysate) at a pH value of 5.1 (Figure 3.3). The samples were therefore transferred into NMR tubes and incubated at 37 °C. All nanogels displayed similar hydrolysis rates in 168 h (Figure 3.3 A).

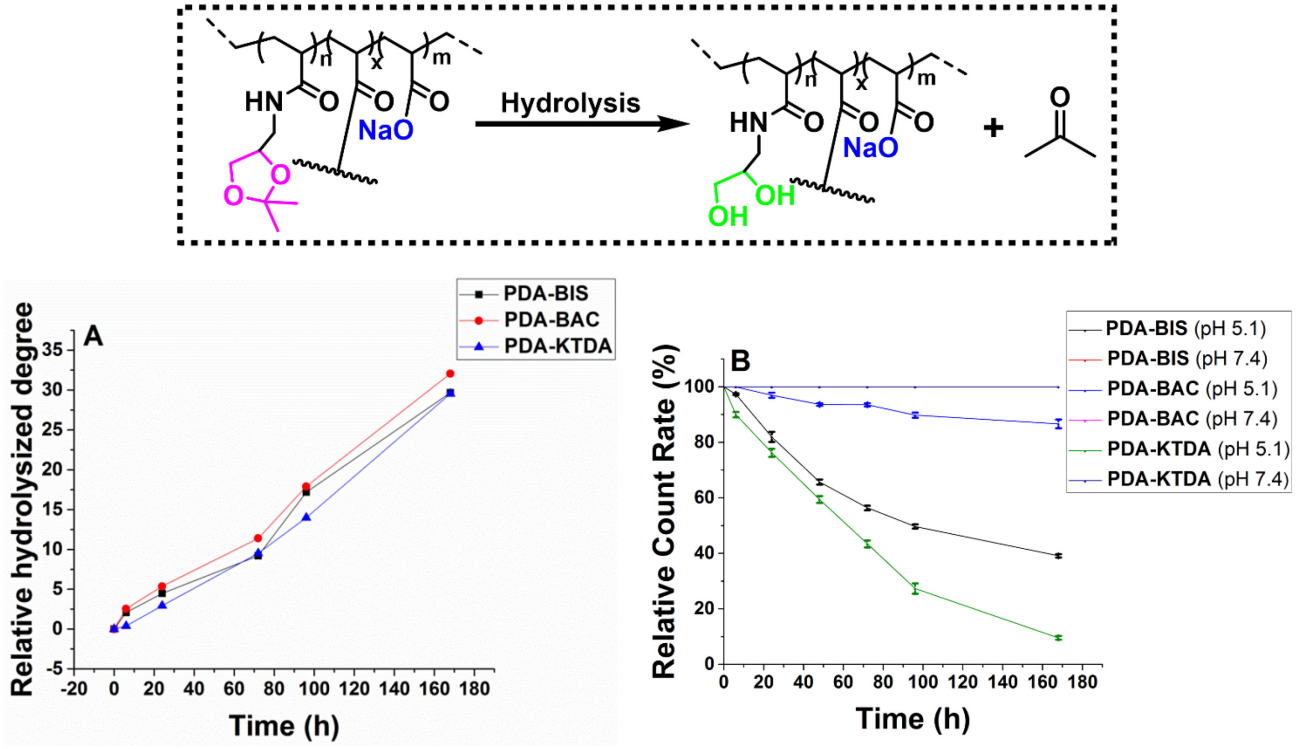

Figure 3.3 Schematic illustration of hydrolysis of nanogels and measurement of the hydrolysis of nanogels at 37 °C at various time points by A) ^{1}H-NMR spectroscopy and B) DLS. All the samples were measured in triplicate. The curves at pH 7.4 in B) are overlapping.

The mean count rate in DLS was further monitored at different pH values and at 37 °C for up to 168 h. All mean count rates remained stable at neutral pH values but decreased at pH 5.1 (Figure 3.3 B). It is well-known that decreased mean count rate measured by DLS refers to lower density nanoparticles.[35, 75] In these nanogels, the degradation of the acetal groups in the nanogels at acidic condition would provide more hydrophilic dihydroxy side groups and, thus, lead to increased swelling of the nanogels and decreased mean count rate. Interestingly, the relative count rate of PDA-BAC (disulfide crosslinker) decreases much slower compared to all other samples which refers to a higher density of crosslinking. Additional thioether bonds might be produced by an attack of radicals on the disulfide bonds during the polymerization.[76, 77] On the contrary, the relative count rate of PDA-KTDA (ketal crosslinker) displayed the fastest decrease due to the disintegration of the crosslinker unit along the hydrolysis of the cyclic acetal groups in DMDOMA repeating units.

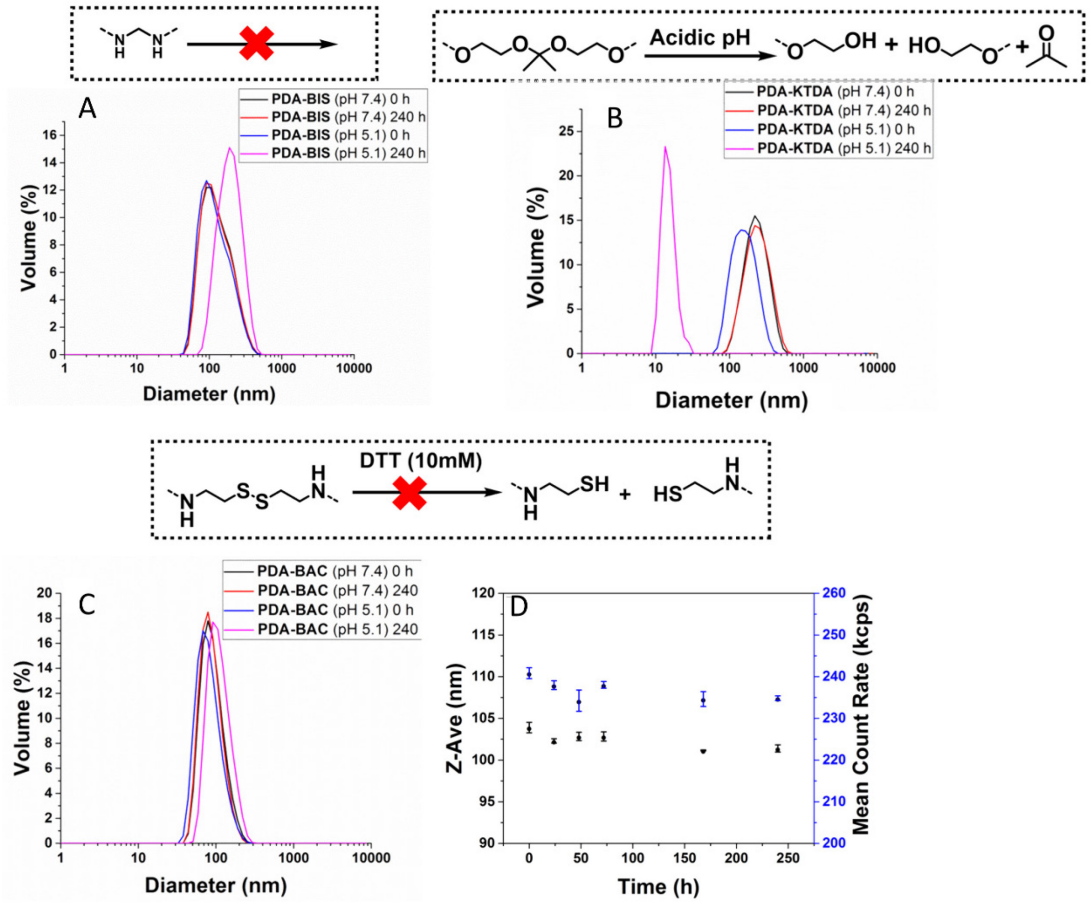

Figure 3.4 Degradation study of A) PDA-BIS, B) PDA-KTDA and C) PDA-BAC at different pH value and E) PDA-BAC with DTT (10 mM) at pH 7.4 using DLS at 37 °C.

Degradation experiments were also investigated by DLS under various conditions. Among them, PDA-BIS was used as a control and incubated at 37 °C and pH 5.1 as well as at neutral conditions (pH 7.4) for 10 days (Figure 3.4 A). Because of the stable crosslinker, the size did not change at neutral conditions but increased at a pH value of 5.1, which is related to the hydrolysis of acetal groups and the subsequent swelling in water. PDA-BAC was also hydrolyzed under the same conditions and displayed similar behavior like PDA-BIS in both cases (Figure 3.4 C), which proves the stability of this crosslinker to acidic conditions. However, it is well-known that disulfide bonds are sensitive to reductive agents such as DTT, GSH and TCEP.[78, 79] To test such a reduction, DTT (10 mM) was added to the PDA-BAC suspension at physiological conditions (pH 7.4, 37 °C) and was then monitored by DLS for 10 days. A stable size and mean count rate was observed (Figure 3.4 D), which is most likely related to the formation of non-cleavable thioether links. PDA-KETA nanogels were also incubated under neutral condition (pH 7.4) and acidic condition (pH 5.1) at 37 °C for 10 days (Figure 3.4 B). The size remained constant at physiological pH value, whereas it decreased to 14 nm in acidic conditions. These results prove that the ketal linkage is sensitive towards acidic cleavage and, thus, facilitates the degradation into single polymer chains.

Nile red was used as a hydrophobic model for the encapsulation and release by the nanogels. Due to the encapsulation of Nile red in hydrophobic domains in the nanogels, a strong fluorescence is observed.[35] When

incubating nanogels loaded with Nile red under neutral conditions (pH 7.4), the intensity of the fluorescence remained stable up to 168 h, while it decreased for all nanogels kept at pH 5.1. The hydrolysis of acetal side groups turns the nanogels more hydrophilic which results in a fluorescence quenching due to increased aggregation of the Nile red (Figure 3.5 A). Among them, the intensity in PDA-KTDA decreased the fastest due to the additional hydrolysis of the ketal group in the crosslinker. In contrast, the slowest decrease occurs for PDA-BAC which is again related to the higher density of crosslinking due to above mentioned thioether links in.

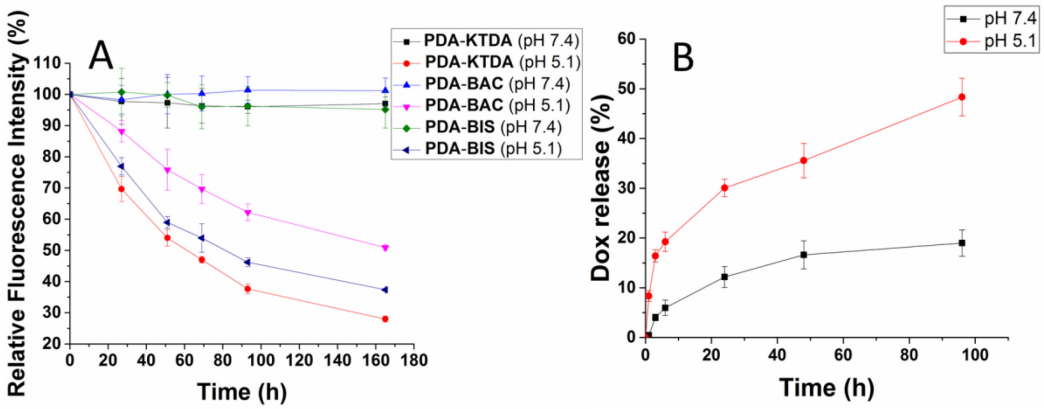

Figure 3.5 A) Change of relative fluorescence intensity over time of different nanogels loaded with nile red and incubated in neutral (pH = 7.4) or acidic buffer (pH = 5.1) at 37 °C, B) Release profile of DOX from DOX-loaded nanogel (PDA-KTDA) at different pH values. The samples were incubated at 37 °C within a dialysis bag, and samples of the outer medium were taken to evaluate the amount of released DOX. Three samples for each pH condition were measured at the same time.

Stimulated by their excellent performance in the encapsulation and release of Nile red, the nanogel were subsequently applied for the transport of an active ingredient. For this purpose, doxorubicin (DOX) was chosen because it is a potent anti-cancer drug that is commonly used for encapsulation experiments, which allows comparability to other systems.[80] DOX was therefore encapsulated into PDA-KTDA, which is the best performing degradable nanogel, in order to investigate its potential for controlled release. The drug loading efficiency (52%) and drug loading content (2.9%) were calculated from calibrated fluorescence intensity measurements. The release experiments revealed that the DOX release was significantly enhanced at pH 5.1, which was up to 50% within four days, while only 20% DOX was released at a pH value of 7.4 within the same time (Figure 3.5 B). Further experiments were performed in cell cytotoxicity tests and revealed that PDA-KTDA represented no cytotoxicity effect on L929 cells after 48 h incubation at concentrations up to 1000 ug mL^{-1} (Figure 3.6 A). In contrast, the DOX-loaded nanogels, as well as free DOX, represented a clear dose and time-dependent decrease in cell viability. The significant reduction of cell viability by the established cytostatic drug DOX was expected, while the enhancement of this effect by the application of DOX-loaded nanogels was somewhat unexpected and could be ascribed to the faster uptake of DOX-loaded nanogels than free DOX which was also reported for other kinds of nanocarriers.[81, 82]

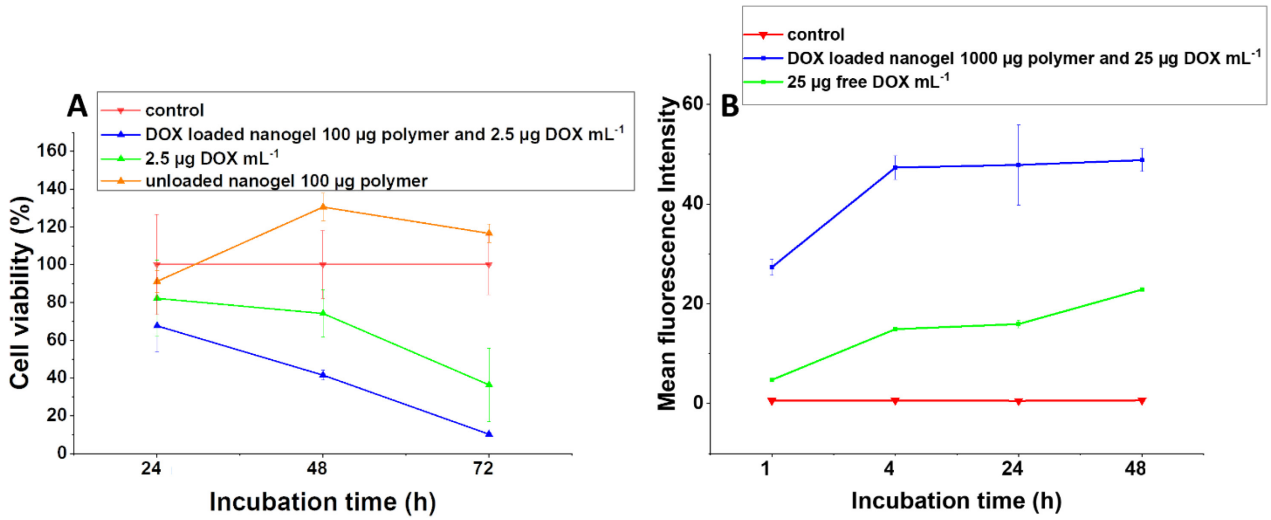

Figure 3.6 A) Cell viability of L929 mouse fibroblasts after incubation with unloaded nanogels (100 µg mL^{-1}), DOX loaded nanogels (100 µg polymer and 2.5 µg DOX mL^{-1}) and free DOX (2.5 µg DOX mL^{-1}) for 24, 48 and 72 h, respectively. Data represent mean values ± SD of 6-fold measured samples. B) Flow cytometry investigation on the time dependent uptake of DOX loaded nanogels (1000 µg polymer and 25 µg DOX mL^{-1}) and the free DOX (25 µg DOX mL^{-1}) by L929 mouse fibroblasts at 37 °C following incubation times of 1, 4, 24 and 48 h. Cells incubated only with culture medium served as control. Line plot depicts mean fluorescence intensities obtained from flow cytometry of the analyzed cell populations. The data are expressed as mean ± SD of triplicate samples.

In order to ensure the assumption, the uptake kinetics of DOX-loaded nanogels were evaluated by flow cytometry in comparison to the free DOX (Figure 3.6 B). The mean fluorescence intensities of the cell populations displayed the time-dependent increase in both DOX-loaded nanogels and free DOX. The DOX entrapped in nanogels was taken up much more efficiently compared to the free drug. These results indicate that the prepared nanogels represent a promising alternative to existing carrier systems for drug delivery applications. The work described in this chapter demonstrates the potential of DMDOMA and the respective nanogels for drug delivery applications. While the basic properties of these nanogels are promising, features such as a more controlled release or active targeting remain a challenge similar to all comparable nanogels.

4. Tunable nanogels constructed via supramolecular host-guest interaction

Parts of this chapter have been published in: **P4)** P. Wei, G. Gangapurwala, D. Pretzel, L. M. Wang, S. Schubert, J. C. Brendel, U. S. Schubert, submitted; **P5)** P. Wei, J. A. Czaplewska, L. M. Wang, S. Schubert, J. C. Brendel, U. S. Schubert, *ACS Macro Lett.* **2020**, *9*, 540-545.

As mentioned before, nanogels made by precipitation polymerization represent an attractive material for drug delivery. A clear limitation, however, remains the sensitivity of the process to changes in composition and structure. In particular deprotonated acrylic acid (carboxylates) plays an important role in stabilization of the resulting nanogels circumventing the use of detrimental surfactants that are difficult to remove. The use of acrylic acid further improves the loading capacities of positively charged drugs such as doxorubicin (DOX) by electrostatic interaction.[33, 83] However, the content of acrylic acid compared to the total amount of monomers has to be kept at around 10% as the hydrophilic character of the acrylic acid prevents a higher amount of incorporation and lower contents would not provide sufficient stability.[33, 37, 38, 84, 85] This limitation also applies for the above-mentioned novel nanogels formed by DMDOMA (Chapter 3).

However, considering the experience on the host-guest interaction with pillar[5]arene and guests in Chapter 2, carboxylate-pillar[5]arene in combination with guests based on pyridinium salts provides a versatile strategy to tune the content of carboxylate in nanogels. In the first part of Chapter 4, the preparation of nanogels based on DMDOMA and comonomers bearing pyridinium units was examined to integrate anker points for subsequent modification by complexation with suitable hosts.

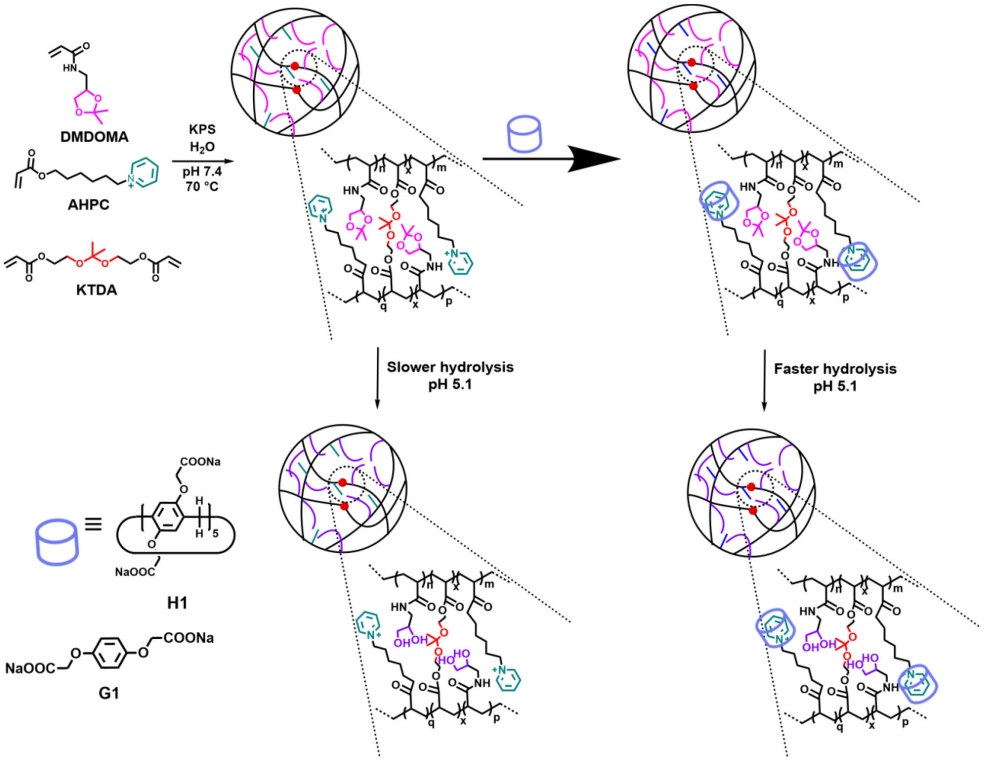

Figure 4.1 Schematic representation of nanogel-preparation, modification with the carboxylate pillar[5]arene, and the respective hydrolysis behavior.

In accordance with the common use of 10% charged acrylic acid for stabilization, a one-to-one replacement with the pyridinium salt-based co-monomer 6-acryloyloxyhexylpyridinium chloride (AHPC) was tested. Surprisingly, this first approach already resulted in the successful formation of stable, positively charged nanogels providing points for interaction with the carboxylate-pillar[5]arene.[86-88] To the best of our knowledge, this is the first report of such a modification with a double-functional comonomer. This successful modification provides a variety of options to modify with other functional pillararenes as well which is utilized in the second part of chapter. As described above, the nanogel was prepared by precipitation polymerization of DMDOMA, AHPC and KTDA as monomer, comonomer and crosslinker, respectively (Figure 4.1). Phosphate buffer solution (pH 7.4) was applied to prevent the hydrolysis of DMDOMA and crosslinker. DLS studies and SEM measurements revealed the successful fabrication of well-defined nanogels (Figure 4.2).

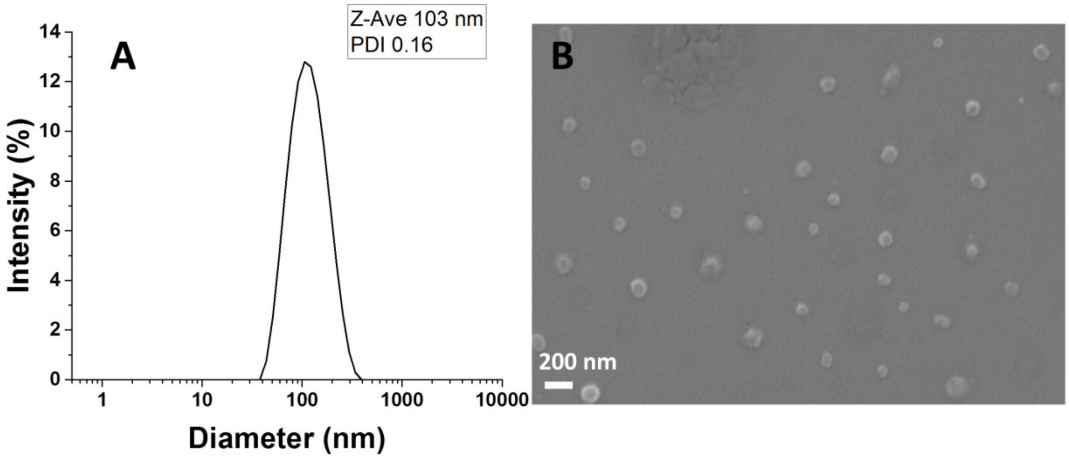

Figure 4.2: A) Size distribution of the nanogels determined by DLS (intensity plot) and B) selected SEM image of a dried sample.

^{1}H NMR spectroscopy was again used to confirm the successful complexation as the presented nanogels were designed to enable the attachment of the carboxylate-pillar[5]arene (**H1**) (Figure 4.3 A, B, C). The disappearance and shift of signals for the pyridinium salts in the nanogels were observed, which are also frequently reported in the literature.[65] The zeta potentials of the nanogels with varying amounts of **H1** were further determined to prove the complexation (Figure 4.3 D). The zeta potential of pure nanogel is about +10 due to the positively charged pyridinium salts, but it rapidly decreases with the addition of **H1** and becomes negative. The zeta potential does not increase significantly when changing the ratio **H1**/pyridinium salt from 0.5 ($\zeta = -21$) to 1 ($\zeta = -23$ mV) or 1.5 ($\zeta = -23.7$ mV). It is assumed that the repulsive coulomb forces between the negatively charged **H1** and the increasingly negatively charged nanogel allow only a small amount of **H1** to form strong complexes with the pyridinium groups.

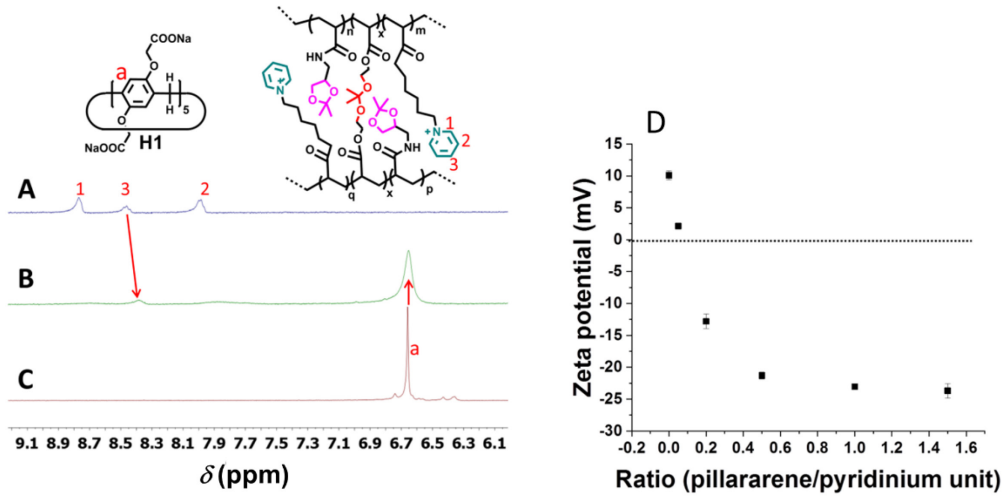

Figure 4.3 ^{1}H NMR (D_2O, 300 MHz) spectra of A) the pure nanogel (5 mg mL^{-1}), B) the nanogel (5 mg mL^{-1}) plus 1 eq. **H1**, C) the pure pillar[5]arene **H1** (3.5 mg mL^{-1}) and D) Zeta potential of the respective nanogels at different ratios of **H1** to pyridinium unit of the nanogel; all measurements were performed in phosphate buffer (pH 7.4, 10 mM) at room temperature.

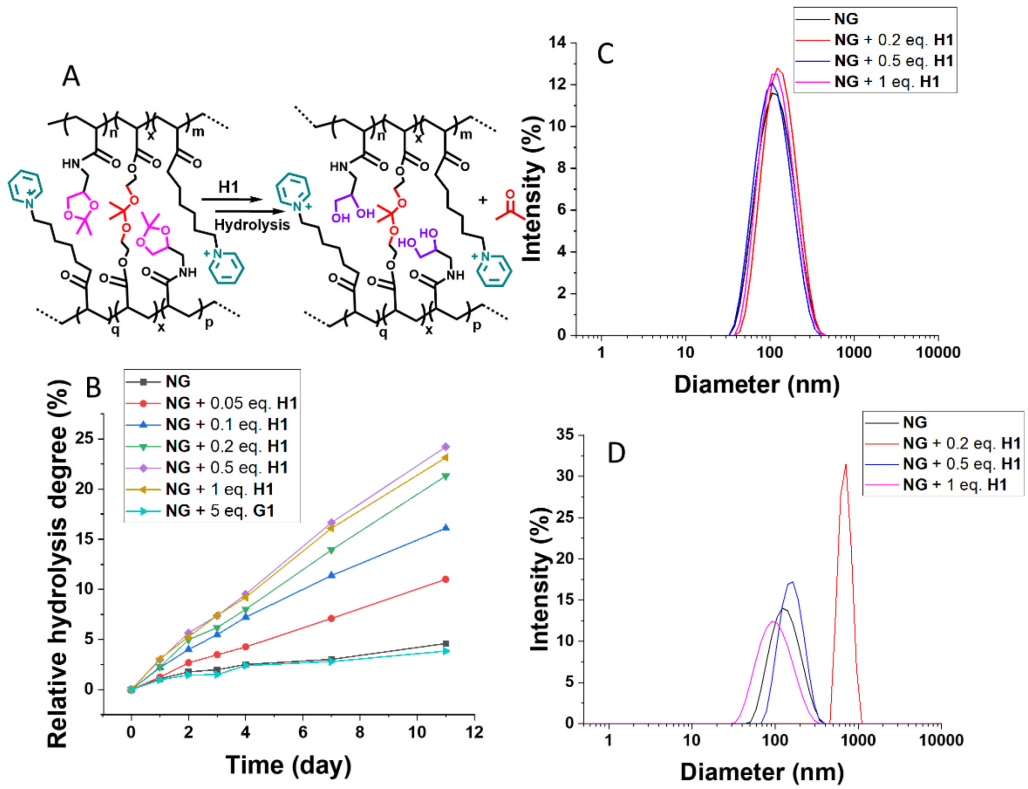

Figure 4.4 A) Schematic representation of hydrolysis of nanogels and B) relative degree of hydrolysis over time for nanogels with different ratios of **H1**; all samples were dispersed in acetate buffer (pH 5.1, 10 mM) and analyzed by ^{1}H NMR spectroscopy (D_2O, 300 MHz). DLS measurements of nanogels with different amounts of **H1** in C) phosphate buffer (pH 7.4, 10 mM) and D) acetate buffer (pH 5.1, 10 mM).

It was already shown that complexed carboxylate-pillar[5]arene (**H1**) can accelerate the hydrolysis rate of linear PDMDOMA in acidic conditions (Chapter 2.2). In case of the presented nanogels the multiple guest moieties

allow the tuning of the **H1** content and, thus, provide the possibility to tune the hydrolysis rate of acetal groups in DMDOMA. To monitor the hydrolysis rate in correlation with the **H1** content, again the signal for the released acetone in the ^{1}H NMR spectra was compared to the acetate signal. All samples were incubated in acetate buffer (pH 5.1) at 37 °C and analysed at different time points over a period of 11 days. As shown in Figure 4.4 A, the hydrolysis rates were significantly enhanced in the presence of higher amounts of **H1**, which is consistent with the previous work. Interestingly, similar rates are observed for 0.5 eq. and 1 eq. **H1**, which confirms the assumption that only a limited amount of **H1** can be incorporated into the nanogel. Additionally, the control experiment using the carboxylate modified repeating unit of **H1** (**H2**) resulted in no difference of the hydrolysis rate compared to pure nanogels. Therefore, it was concluded that the host-guest interaction plays an essential role in the acceleration of the hydrolysis. It is well-known that carboxylate groups can reversibly be protonated within a physiological relevant range regarding their typical pK_a values of around 5.[89] For nanogels, the protonation at low pH values renders the materials more hydrophobic and, thus, leads to a shrinkage of the nanogels. The given nanogels with various amounts of carboxylate-pillar[5]arene (**H1**) were therefore tested for changes in diameters (determined by DLS) at different pH values (Figure 4.4 C, D). All the nanogels are stable at neutral conditions (pH 7.4) due to the help of pyridinium salts or the excess of carboxylate groups. However, their size changed significantly in pH 5.1 depending on the ratios of **H1**. The NG with 1 eq. **H1** demonstrated the best stability in these acidic conditions, while the nanogel with 0.5 eq. **H1** already resulted in a slight increase in size. For 0.2 eq. of **H1**, aggregate occurred rather rapidly at the reduced pH value. This difference in the behavior is further supporting the theory that the characteristics of the nanogel can be tuned by the supramolecular interaction, and it can be assumed that the aggregation in nanogels with 0.2 eq. **H1** refers to the partial protonation of the carboxylate groups and, thus, results in a zero net charge of the overall nanogels. Consequently, no repulsive forces remain, which could stabilize the suspension.

Table 4.1 Characterization of DOX-loading efficiency by different encapsulated pathway.

Abbrev.	DOX/NG (wt. %)	DLC (wt. %)	EE (wt. %)
NG	5.8	1.6	30.8
NG + 1 eq. **H1**	5.8	3.4	58
NG + 1 eq. **H1**	11.6	7.8	67
NG + 1 eq. **H1**	17.4	14.2	81.7
NG + 1 eq. **H1**	23.3	16	69

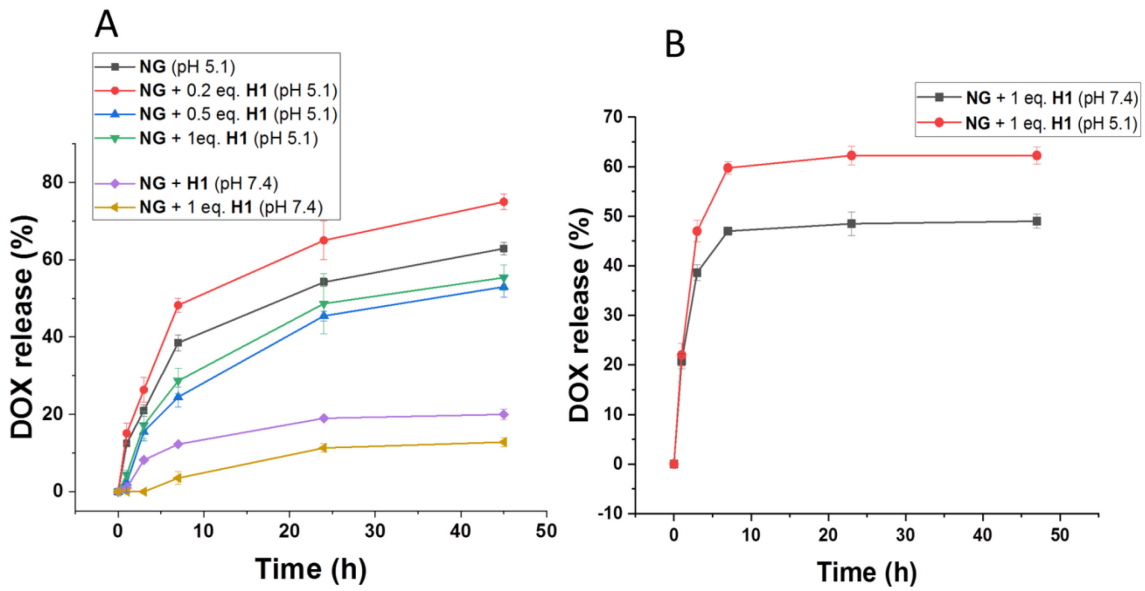

Figure 4.5 Release of DOX over time from A) loaded nanogels modified with varying amounts of **H1**, B) loaded nanogels + 1 eq. **H1** (DLC: 16 wt %) at different pH values.

DOX was again used as a model drug with its beneficial optical properties to evaluate the nanogels as potent drug delivery systems. The limited solubility in water at physiological conditions as well as its positive charge in the physiological pH range allows DOX to be encapsulated in nanocarriers by hydrophobic interaction with the acetal based polymer or by electrostatic interaction.[70, 90, 91] Therefore, the positively charged, pure nanogels (NG) and the negatively charged nanogels modified with 1 eq. **H1** (NG + 1 eq. **H1**) were studied regarding their loading capacity. As listed in Table 4.1, nanogels with 1 eq. **H1** enhance the loading capacity compared to the pure nanogels. Additionally, a significantly increase in encapsulation efficiency can be observed with addition of increasing amounts of DOX to the modified nanogel, which underlines the importance of electrostatic interactions for the encapsulation. A maximum EE of 81.7 wt% is reached when applying 17.4 wt% of DOX/NG in the initial solution. The initial increase with additional DOX in the solutions may be attributed to the low overall concentrations and the remaining solubility of DOX in water. In the subsequent release experiments, varying amounts of **H1** were added to nanogels previously loaded with DOX (DLC = 16 wt%) to guarantee comparable starting conditions (Figure 4.5 A). At physiological conditions, only 10% or 16% of DOX were released from all the nanogels, which is ascribed to the stable acetal groups at the conditions holding the drug within the more hydrophobic environment, whereas very different release behavior was observed in acidic conditions. The DOX release from nanogels with 0.2 eq. **H1** was fastest due to the precipitation of these nanogels in pH 5.1 as mentioned above. The lower release rates for nanogels with 0.5 and 1 eq. of **H1** compared to the pure nanogel could be related to the remaining negative charges in nanogels, which can interact with the positively charged DOX. It is well-known that DOX has a better water-solubility in acidic conditions due to the protonated amino groups.[92, 93] This effect can explain the intermediate release rates from pure nanogels with 0.2 eq. **H1** and 0.5 eq. **H1**. Nevertheless, the observed rates are still much faster than at neutral conditions. In addition, the release of modified nanogels (1 eq. **H1**) with the highest degree of drug loading (16 wt%) was

examined (Figure 4.5 B). In this case, a burst release was observed initially independent of the conditions, which might be related to the dilution and is reported similarly in literature.[33] Nevertheless, the release is still considerably higher at acidic conditions (62% in acidic condition *vs.* 47% in natural condition). Stability studies revealed that only nanogels with 1 eq. **H1** was stable in cellular media without serum. Therefore, the cytotoxicity experiments were limited to these nanogels, but demonstrated a good biocompatibility of the pure materials, while the DOX-loaded nanogels (1 eq. **H1**) revealed a time-dependent decrease of viability.

It is well-known that the often hydrophilic surface of nanogels, such as modified PEG or carboxylate groups, may cause poor cellular interaction and, thus, limits the uptake and the efficiency of drug-loaded nanogels.[94, 95] In order to treat this problem, active targeting strategies were developed relying on the covalent modification with targeting units, which have to be introduced post-polymerization to the nanogels or by integrating targeting monomers.[96, 97] However, both approaches require additional synthetic efforts and are limited in terms of targeting units. Supramolecular chemistry opens possibilities to overcome these limitations and facilitate a more versatile access to this strategy.

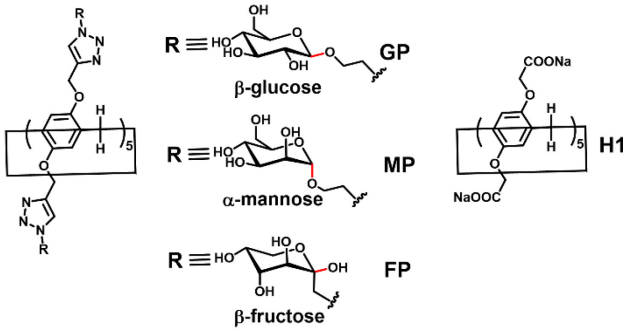

Figure 4.6 Schematic representation of pillar[5]arene derivatives: β-Glucose-pillar[5]arene (GP), *α*-mannose-pillar[5]arene (MP), *β*-fructose-pillar[5]arene (FP), and carboxylate-pillar[5]arene (**H1**, synthesized as reported in Chapter 2.2).

As described in the previous work, suitable guest moiety can be integrated into the nanogels based on DMDOMA which allows the complexation with pillar[5]arene molecules by supramolecular interactions. Following a similar strategy, the well-studied targeting units glucose, mannose and fructose were first connected with pillar[5]arenes using alkyne-azide cycloaddition click reactions (Figure 4.6).[98-100] Consequently, the sugar modified pillar[5]arenes were investigated for their ability to form complexes with the nanogels. In the ^{1}H NMR spectra, peaks of pyridinium salts in nanogels disappeared or shifted, which were same to the phenomenon in the first part chapter (Figure 4.3) and confirmed the successful complexation. In addition, Concanavalin A (Con A) was chosen to prove the selective binding of the modified nanogels. The homotetramer of Con A represents four sites for binding to *α*-D-mannosyl- and *α*-D-glucosyl residues.[41] The addition of Con A to nanogels modified with the respective sugars should consequently result in crosslinking and precipitation while it should not affect the other nanogels.[101] The diameters of MP-NG (α-mannose-pillar[5]arene nanogel) rapidly increased with the addition of Con A, while other nanogels displayed no change(Figure 4.7). Although glucose is often reported to bind to Con A, the lack of any changes in the case of glucose-pillar[5]arene modified nanogels is

most likely related to the β-anomer, which was used and does not interact with Con A. Furthermore, the scattering intensity remained stable after adding Con A into the pure solution of MP, but without nanogels, which proves the robustness of the complexes between the sugar-modified pillar[5]arene and the nanogel.

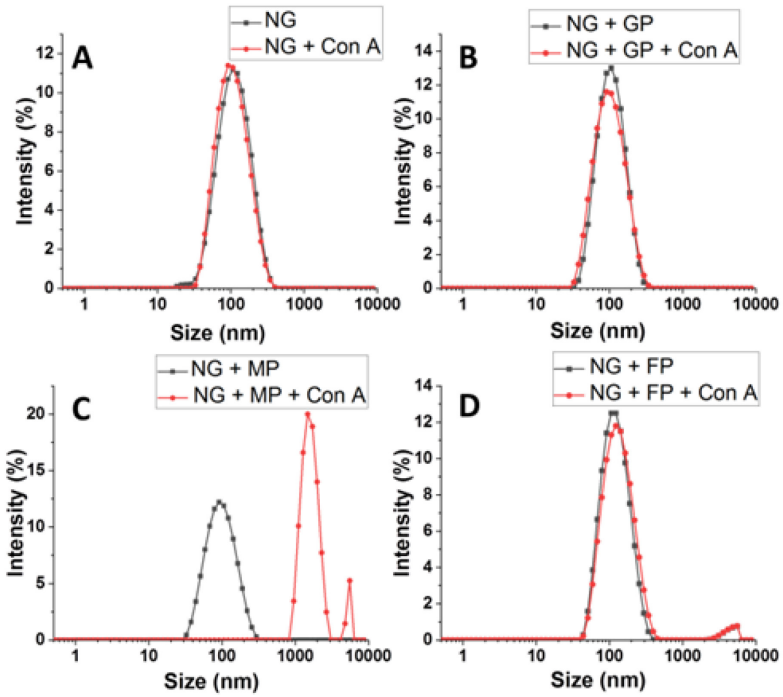

Figure 4.7: Selective binding studies of nanogels with and without sugar modified pillar[5]arene and Con A in a buffer solution (10 mM, pH 7.4, 0.1 mM $CaCl_2$ and $MnCl_2$) measured by DLS at room temperature.

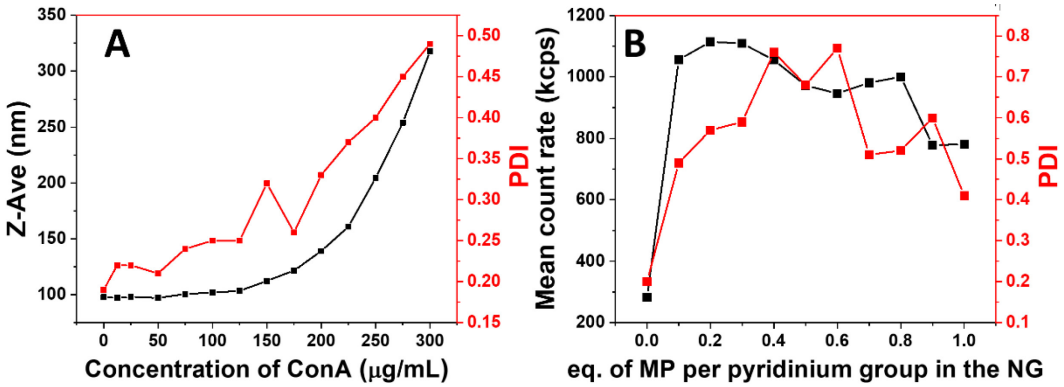

Figure 4.8: DLS measurement of A) MP-NG (1 mg mL^{-1}) at different Con A concentrations and B) NG (1 mg mL^{-1}) with variation of MP equivalents by fixed Con A concentration (0.3 mg mL^{-1}).

Further variation of the added Con A amount revealed a clear dependency of the aggregation behavior on the concentration using the MP-NG (Figure 4.8 A). Precipitation only occurred with high amounts of Con A. Another interesting parameter is the density of MP on the nanogels and its effect on the binding ability (Figure 4.8 B). The results demonstrate that even low amounts of MP (0.1 eq. per pyridinium group in the nanogels) in the nanogels resulted in aggregation at a constant concentration (0.3 mg mL^{-1}). of Con A. A further increase of

MP, however, did not cause a significant change of the aggregation tendency. This result highlights the good accessibility of the mannose groups on the surface of the nanogels and opens the possibility for introducing additional targeting units attached to pillararenes.

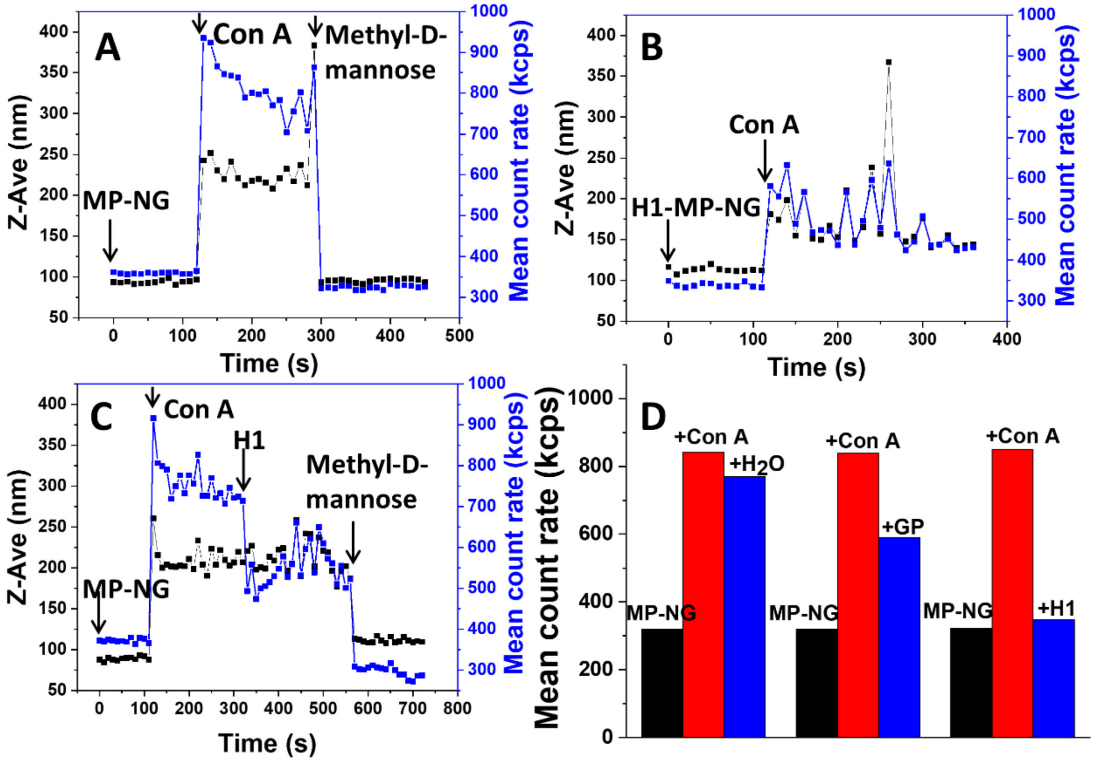

Figure 4.9: Competition experiments using DLS: A) MP-NG, addition of Con A, then addition of methyl-D-mannose; B) MP-H1-NG , addition of Con A; C) MP-NG, addition of Con A, addition of **H1** (1 eq. compared to MP), addition of methyl-D-mannose; D) MP-NG, addition of Con A, addition of excess **H1** (2 eq. compared to MP), GP (2 eq. compared to MP) or same amount of pure H_2O.

As known from literature reports, free methyl-D-mannose has a strong binding ability with Con A and can, therefore, be used to interfere with the binding of Con A with other sugar derivatives by competition.[102] Thus, competition experiments were performed between the free methyl-D-mannose and MP-NG. Methyl-D-mannose is known for its strong interaction with Con A surpassing most other sugar derivatives. Consequently, previously aggregated nanogels can be disassembled by adding an excess methyl-D-mannose (Figure 4.9 A). In further experiments, the dynamic exchange and competition between different pillar[5]arenes was evaluated.[20, 103] As an example, carboxylate-pillar[5]arene (**H1**) forms stronger complexes with pyridinium salts compared to non-charged pillar[5]arenes due to additional electrostatic interactions as they are described in the previous Chapter 2.2.[20, 100] To demonstrate the influence of this additional interaction, carboxylate-pillar[5]arene (**H1**) was mixed with MP-NG and added to Con A. A clear decrease in the mean count rate was observed compared to the values obtained in the previous experiment for the pure mannose-pillar[5]arene (Figure 4.9 B). To go a step further, MP-NG was first mixed with Con A to yield aggregation (Figure 4.9 C), and subsequently the **H1** (1 eq. compared to MP) was added into the solution. A clear decrease of the mean count rate could be observed by DLS (Figure 4.9 B) and the final values was in same range as the previous experiment. As a consequence, the

interaction between the MP and the nanogel must be dynamic, and the addition of **H1** results in a partial exchange of the complexes between the pillararenes and the pyridinium groups on the nanogel. Nevertheless, only the final addition of methyl-D-mannose results in a full disintegration of remaining aggregates, which confirms a residual interaction of the modified nanogels with the Con A and contradicts a full replacement of the MP by the **H1** on the nanogels. In order to induce a clearer shift in the potential equilibrium, an excess of **H1** (2 eq. compared to MP) was added in a further competition experiment. The mean count rate of the MP-NG mixed with Con A almost returns to the initial stage with the added excess of **H1** confirming a shift in the equilibrium and an almost full exchange of the mannose-pillararene complexes (Figure 4.9 D). In addition to the charged **H1**, GP (2 eq. compared to MP) was applied in these competition experiments using similar conditions. Still a considerable decrease of the count rate is observed (Figure 4.9 D), however it does not result in a full reversion of the aggregation as for the experiment with **H1**. These findings confirm that the previously mentioned complexation strength is the key parameter as it is higher for the **H1** due to additional electrostatic interactions. Overall, this work represents a straightforward method to reversibly introduce various targeting units to the nanogels by supramolecular host-guest interaction and promises convenient access to adaptable nanostructures for the fast screening of targeted delivery systems

5. Summary

In recent years, the fast development of the supramolecular host-guest interactions has attracted significant attention. Based on the dynamic interaction between macrocyclic structures and a variety of matching guests, two chemical entities can be conveniently bond together without the need for further reactions. These features also facilitate straightforward modifications of polymers to create more advanced materials with tunable properties or additional functionalities. Most commonly cyclodextrins have been used as supramolecular hosts and opened up exiting new opportunities in the field of polymer chemistry. More recently, a new generation of macrocycles, the pillar[n]arenes was established which not only broadened the range of guests but also allowed the facile modification of their exterior. The early reports on these materials certainly prove their potential, however several aspects concerning the building of polymer nanostructures based on such supramolecular host-guest complexes, as well as their therapeutic applications as smart nanocarriers remain unexplored.

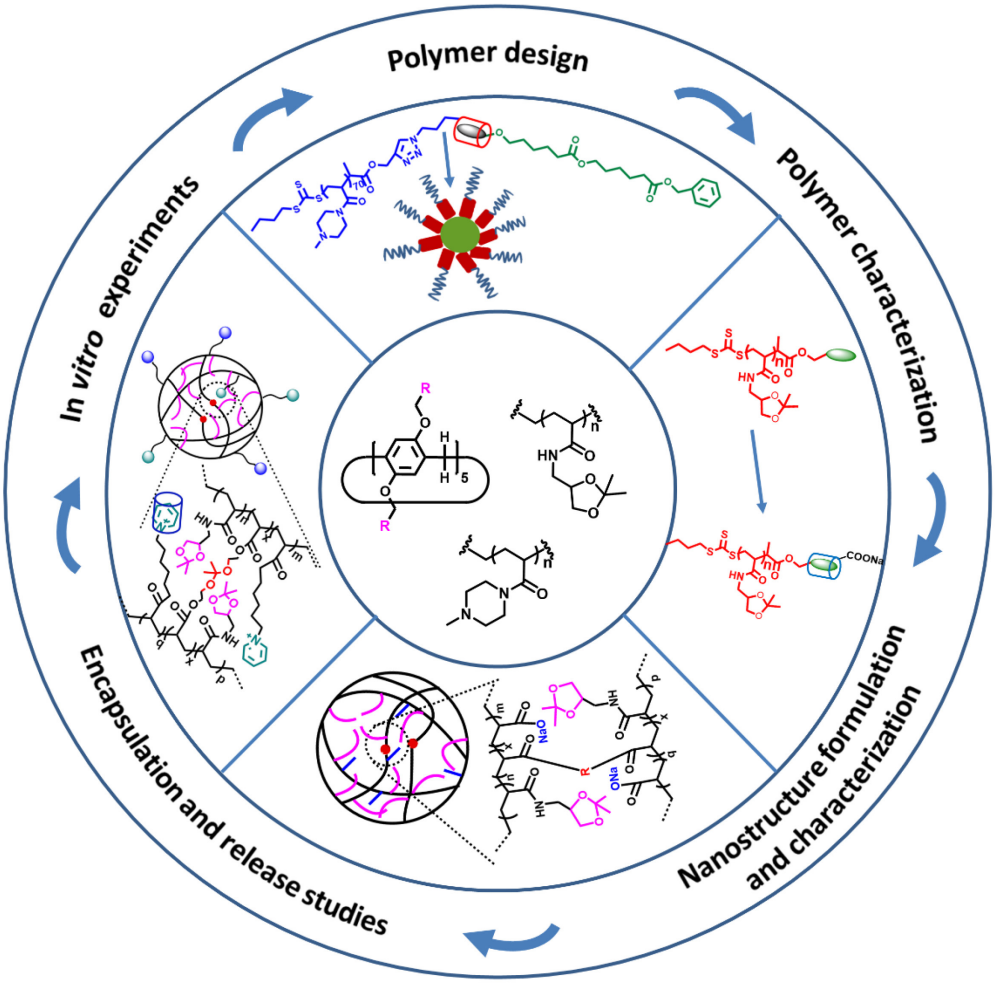

Figure 5.1 Schematic representation of the workflow, polymers and polymer nanostructures used in the represented studies in this thesis.

The presented thesis aims to investigate several strategies based on the above-mentioned interactions and materials. It is subdivided into chapters on (1) the formation of quasi-block copolymers that are constructed *via* host-guest interaction of water-soluble pillar[5]arene based polymers and guest modified hydrophobic polymers,

which were assembled into nanoparticles, (2) the investigation of thermoresponsive polymers (PDMDOMA) modified with pillar[5]arenes to tune their LCST behavior and hydrolysis rate, (3) the formulation and characterization of degradable nanogels prepared from above the above mentioned thermoresponsive polymers for encapsulation and release of chemotherapeutic (doxorubicin: DOX) and, finally, (4) the integration of supramolecular host-guest interactions into nanogels for the tuning of encapsulation and release of DOX and the introduction of targeting units at the surface of these nanogels (Figure 5.1).

In the Chapter 2.1, amphiphilic quasi-block copolymers were prepared by combination of pillar[5]arene-modified hydrophilic polymers and viologen-polycaprolactones (viologen-PCL). Stability measurement of the obtained nanostructures in phosphate buffer and cellular media revealed that only the slightly charged poly(*N*-acryloyl-*N*'-methyl piperazine) (PNAMP) gave stable nanostructures in both conditions. Unexpectedly, also larger but stable nanoparticles were obtained for the non-modified PCL, which is not capable of forming a host-guest inclusion with the pillar[5]arene modified PNAMP. Both materials response to decreasing pH value due to the tertiary amino groups in the PNAMP. Interestingly, the comparison of both materials revealed that the enzymatic degradation of the PCL in acidic conditions can be retarded by the presence of the host-guest complex. Furthermore, the mixing of viologen modified PCL with non-modified polymer enabled access to more controlled degradation rates, which are in between the pure materials.

Parallel to the above mentioned studies, the effect of such host-guest interactions on the properties of thermoresponsive and degradable polymers were evaluated. The studies revealed that functional pillar[5]arenes can be utilized not only to tune the LCST behavior of the thermoresponsive polymer poly(*N*-[(2,2-dimethyl-1,3-dioxolane)-methyl]acrylamide) (PDMDOMA) but also selectively accelerate the hydrolysis of the cyclic acetal groups under acidic condition. Therefore, the molar masses of the polymers and their combination with two different pillar[5]arenes bearing either oligoethylene oxide or carboxylate groups were varied systematically. The complex formation led to an increase of the LCST of the polymer whereat carboxylate functionalized pillar[5]arenes which are negatively charged caused a stronger shift than the respective oligoethylene oxide modified ones. Further investigations on this complex revealed that the acidic hydrolysis of the acetal groups in the polymer can be accelerated in the presence of the carboxylate based pillar[5]arene, which was not the case for the oligoethylene oxide modified system or a structurally similar carboxylic acid lacking the ability to form a complex.

Based on these interesting results on the LCST and pH-sensitive behavior of PDMDOMA, a new degradable nanogel was designed utilizing precipitation polymerization as a straightforward and versatile preparation technique. Well-defined nanogels were obtained and degradation studies confirmed the previous observed sensitivity towards acidic conditions, which cause a complete degradation depending on the cross-linker but maintain good stability in a neutral physiological environment. The best performing system was studied regarding its release kinetics using the anticancer drug doxorubicin (DOX) as a model drug, which confirmed a selective release in acidic conditions. *In vitro* studies based on the latter system revealed excellent

biocompatibility of the unloaded nanogel and the capability of the DOX-loaded nanogel to mediate cytotoxic effects in a concentration and time-dependent manner with an even higher efficiency than the free drug.

Due to limited adjustment possibilities in the content of carboxylate groups in the above mentioned nanogels, supramolecular modifications were considered using carboxylate-pillar[5]arene, as it was previously demonstrated for the linear polymers. As a consequent, the stabilizing acrylic acid was replaced with pyridinium salts as comonomer in the DMDOMA nanogels which enable the formation of host-guest interaction with pillar[5]arenes. Due to the strong complex between the pyridinium salt and the carboxylate-pillar[5]arene (**H1**), the surface charge on the nanogels can gradually be converted from positive to negative with the amount of added **H1**. Similar to the linear polymer, the hydrolysis rate of the acetal group on the nanogels could be promoted by the presence of **H1**. Control experiments using the aromatic carboxylate units (a subunit of the macrocycle **H1**) prove that the hydrolysis is only affected in case of the host-guest interaction. In addition, the variation of the negative charge density in the nanogel enables a tunable encapsulation of DOX with different ratios of the **H1**. The consecutive release experiments further support an excellent tuneability of the nanogel release profile in acidic conditions while almost no release is observed at physiological conditions. First cell experiments demonstrated that the DOX-loaded nanogels are cytotoxic and taken up by cells, while no toxicity is observed for the pure nanogels.

In addition to this tuning of the nanogel properties, the surface modification of the nanogels with targeting units was envisaged by the pillar[5]arene based host-guest interaction. This approach enabled the attachment of various carbohydrate units by simply adding the respectively modified pillar[5]arene host molecules to the nanogel suspension. The dynamic interaction facilitated the reversible exchange of these units, which allowed the statistical combination of various functional moieties. Within this thesis, the mannose-modified host material revealed that an excellent selectivity for the lectin Concanavalin A (Con A) can be achieved comparing it to various other sugar-modified host materials. The straightforward attachment circumventing tedious chemical reactions for modifications represents a unique pathway for the fast screening of not only single targeting moieties but multiple units even comprising further functionalities to find optimized combinations.

In summary, this thesis demonstrates the tremendous potential of supramolecular modifications based on pillar[5]arenes for the advancing existing polymer designs and nanostructures. It comprises a detailed characterization of all prepared nanostructures, provides novel aspects to the formulation of smart nanostructures, and examines their suitability for encapsulation and release of pharmaceutical active ingredients.

6. Zusammenfassung

In den letzten Jahren hat die Entwicklung von supramolekularen Wirt-Gast-Interaktionen zunehmende Bedeutung erlangt. Basierend auf der dynamischen Interaktion zwischen makrozyklischen Strukturen und einer Vielzahl von passenden Gastmolekülen können zwei chemische Gruppen direkt ohne chemische Reaktion miteinander verbunden werden. Diese Besonderheit ermöglicht die einfache Modifikation von Polymeren um neuartige Materialien mit einstellbaren Eigenschaften bzw. besonderen Funktionen zu schaffen. Bisher wurden am häufigsten Cyclodextrine als supramolekulare Wirtstrukturen verwendet, welche viele neue Möglichkeiten auf dem Gebiet der Polymerchemie eröffneten. In letzter Zeit wurde eine neue Generation von Makrozyklen, sogenannte Pillar[n]arene, entwickelt, die nicht nur ein breiteres Spektrum von Gastmolekülen eröffnet, sondern auch eine leichtere Modifikation ihrer äußeren chemischem Struktur ermöglicht. Die ersten Berichte über diese Materialien belegen zweifellos deren Potenzial, doch einige Aspekte in Bezug auf polymere Nanostrukturen basierend auf solchen supramolekularen Wirt-Gast-Komplexen, sowie ihre anschließende Anwendung als intelligente Nanotransportsysteme für Therapeutika sind bisher weitestgehend unerforscht.

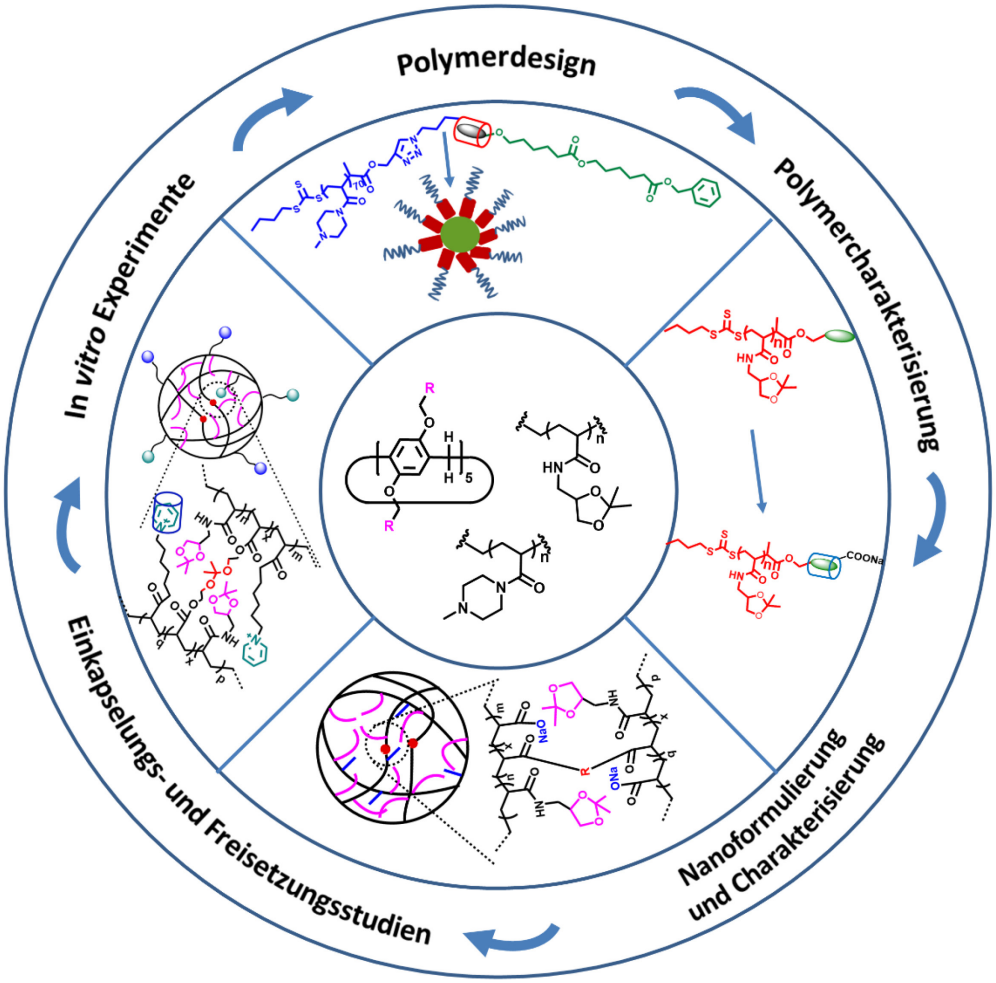

Abbildung 6.1 Schematische Darstellung des Arbeitsablaufs, der Polymere und der Polymernanostrukturen der in dieser Arbeit durchgeführten Studien.

Die vorliegende Arbeit hat das Ziel, eine Reihe an Strategien zu verfolgen, die auf den oben erwähnten Interaktionen und Materialien basieren. Sie gliedert sich in folgende Kapitel: (1) Die Bildung von Quasi-Blockcopolymeren, die durch Wirt-Gast-Interaktion von wasserlöslichen Polymeren auf Basis von Pillar[5]arenen und hydrophoben, mit Gastmolekülen modifizierten Polymeren aufgebaut werden, um Nanopartikel herzustellen, (2) die Untersuchung von thermoresponsiven Polymeren (PDMDOMA), die mit Pillar[5]arenen modifiziert sind, um ihr LCST- und Hydrolyseverhalten zu steuern, (3) die Herstellung und Charakterisierung von abbaubaren Nanogelen, die aus den oben genannten thermoresponsiven Polymeren hergestellt und für Verkapselung und selektive Freisetzung von Chemotherapeutika (z.B. Doxorubicin: DOX) verwendet werden sowie, (4) die Integration von supramolekularen Wirt-Gast-Wechselwirkungen in den entsprechenden Nanogelen zur Steuerung der Einkapselung und Freisetzung von DOX, sowie die Einführung von Targeting-Einheiten an der Oberfläche dieser Nanogele (Abbildung 6. 1).

In Kapitel 2.1 wurden amphiphile Quasi-Blockcopolymere durch Kombination von Pillar[5]aren-modifizierten hydrophilen Polymeren und Viologen-modifizierten Polycaprolactonen (Viologen-PCL) hergestellt. Stabilitätsmessungen der erhaltenen Nanostrukturen in Phosphatpuffer und Nährmedien ergaben, dass nur das schwach geladene Poly(*N*-acryloyl-*N*′-methylpiperazin) (PNAMP) unter den genannten Bedingungen stabile Nanostrukturen ergab. Unerwarteterweise wurden zwar größere, aber auch stabile Nanopartikel für das nicht modifizierte PCL erhalten, welches nicht in der Lage ist Wirt-Gast-Komplexe mit dem Pillar[5]aren-modifizierten PNAMP zu bilden. Beide Materialien reagieren aufgrund der tertiären Aminogruppen im PNAMP auf einen sinkenden pH-Wert. Interessanterweise zeigte der Vergleich beider Materialien, dass der enzymatische Abbau des PCL unter sauren Bedingungen im Falle des stabilen Wirt-Gast-Komplexes verlangsamt werden kann. Darüber hinaus ermöglichte eine Mischung von Viologen-modifiziertem mit nicht modifiziertem PCL eine Steuerung der Abbauraten innerhalb der Grenzen der reinen Materialien.

Parallel zu den oben erwähnten Studien wurde der Einfluss solcher Wirt-Gast-Interaktionen auf die Eigenschaften thermoresponsiver und abbaubarer Polymere untersucht. Die Studien zeigten, dass funktionelle Pillar[5]arenen nicht nur zur Anpassung des LCST-Verhaltens des thermoresponsiven Polymers Poly(*N*-[(2,2-dimethyl-1,3-dioxolan)-methyl]acrylamid) (PDMDOMA) eingesetzt werden können, sondern auch die Hydrolyse der zyklischen Acetalgruppen unter sauren Bedingungen beschleunigen. In Folge wurden systematisch die Molmassen der Polymere variiert und entsprechende Kombination mit zwei unterschiedlichen Pillar[5]arenen, die entweder Oligoethylenoxid- oder Carboxygruppen tragen, untersucht. Die Komplexbildung ergab eine Anhebung der LCST des Polymers, wobei negativ geladene Carboxy-funktionalisierte Pillar[5]arene einen stärkeren Einfluss hatten als die entsprechenden Oligoethylenoxid-modifizierten Pillar[5]arene. Weitere Untersuchungen zu diesem Komplex ergaben, dass die saure Hydrolyse der Acetalgruppen im Polymer in Gegenwart des Carboxy-funktionalisierten Pillar[5]arenes beschleunigt werden kann, was im Falle des Oligoethylenoxid-modifizierten Systems oder bei einer strukturell ähnlichen Carbonsäure, die nicht in der Lage ist Komplexe zu bilden, nicht der Fall war.

Basierend auf diesen Ergebnissen zum LCST-Verhalten und der Sensitivität gegenüber pH-Wertänderungen des untersuchten PDMDOMA wurde ein neuartiges abbaubares Nanogel entwickelt, das mittels Fällungspolymerisation, einer unkomplizierten und vielseitigen Präparationstechnik, hergestellt wurde. Definierte Nanogele wurden erhalten und Abbaustudien bestätigten die zuvor beobachtete Sensitivität gegenüber sauren Bedingungen des Polymers, die je nach Art der Vernetzung auch einen vollständigen Abbau erlaubt, während in neutralen physiologischen Umgebungen eine gute Stabilität garantiert ist. Das vielversprechendste System wurde schließlich hinsichtlich seiner Freisetzungskinetik untersucht, wobei das Krebsmedikament Doxorubicin (DOX) als Modellarzneimittel verwendet wurde. Auch hier konnte eine selektive Freisetzung unter sauren Bedingungen bestätigt werden. *In-vitro*-Studien auf Basis des letztgenannten Systems ergaben eine ausgezeichnete Biokompatibilität des unbeladenen Nanogels, während das DOX-beladenen Nanogel eine zytotoxische Wirkung in Abhängigkeit von Konzentration und Zeit zeigte. Die Effizienz des beladenen Nanogels übertraf hier sogar das freie Medikament.

Aufgrund des begrenzten Spielraums beim Anteil der Carboxygruppen in den oben genannten Nanogelen wurden supramolekulare Modifikationen mit Hilfe des bereits genannten Carboxy-Pillar[5]aren herangezogen, entsprechend dem zuvor berichteten linearen Polymer. Hierfür wurde die stabilisierende Acrylsäure in den DMDOMA-Nanogelen mit Comonomer auf Basis von Pyridiniumsalzen ersetzt, die eine Bildung von Wirt-Gast-Komplexen mit entsprechenden Pillar[5]arenen ermöglichen. Aufgrund der starken Wechselwirkung zwischen dem Pyridiniumsalz und dem Carboxy-Pillar[5]aren (**H1**) kann die Oberflächenladung der Nanogele mit der zugesetzten Menge an **H1** schrittweise von positiv zu negativ gewandelt werden. Ähnlich dem linearen Polymer ist die Hydrolyserate der Acetalgruppe innerhalb des Nanogels bei Anwesenheit von **H1** beschleunigt. Kontrollexperimente mit entsprechenden aromatischen Carboxy-funktionalisierten Untereinheiten des Makrozyklus **H1** beweisen, dass die Hydrolyse nur im Falle intakter Wirt-Gast-Wechselwirkung beeinflusst wird. Darüber hinaus erlaubt die Anpassung der Ladungsdichte im Nanogel eine variable Einkapselung von DOX mit unterschiedlichen Mengen an eingesetztem **H1**. Die folgenden Freisetzungsexperimente belegen darüber hinaus gut steuerbare Freisetzungsraten mit den unterschiedlichen Nanogelen unter sauren Bedingungen, während unter physiologischen Bedingungen in keinem Fall eine signifikante Freisetzung beobachtet wird. Erste Zellexperimente beweisen, dass die DOX-beladenen Nanogele zytotoxisch sind und von den Zellen aufgenommen werden, während im Falle der reinen Nanogelen keine Toxizität beobachtet wird.

Neben der genannten Anpassung der Nanogeleigenschaften war ein weiteres Ziel die Oberflächenmodifikation der Nanogele mit Targeting-Einheiten mittels Wirt-Gast-Interaktion basierend auf Pillar[5]arenen. Dieser Ansatz ermöglicht den einfachen Einbau verschiedener Kohlenhydratstrukturen durch Zugabe der entsprechend modifizierten Pillar[5]arene. Die Dynamik der Interaktion erlaubt zudem einen reversiblen Austausch dieser Einheiten, was die statistische Kombination verschiedener funktioneller Einheiten ermöglicht. Im Rahmen dieser Arbeit zeigte das Mannose-modifizierte Pillar[5]arene eine ausgezeichnete Selektivität bei der Bindung zum Lektin Concanavalin A (ConA) im Vergleich zu den anderen zuckermodifizierten Wirtmolekülen. Die einfache Anbindung an die Nanogele ohne weitere chemische Reaktionen stellt eine einzigartige Modifikationsmöglichkeit dar, die ein schnelles Screening verschiedenster Bausteine eröffnet, bei dem nicht nur

einzelne Targetinggruppen getestet werden können, sondern mehrerer Einheiten mit verschiedenen Funktionalitäten kombiniert werden können, um optimale Zusammensetzungen zu finden.

Zusammenfassend verdeutlicht diese Arbeit das enorme Potenzial, das supramolekulare Modifikationen auf Basis von Pillar[5]arenen für die Weiterentwicklung bestehender Polymermaterialien und Nanostrukturen besitzen. Neben einer detaillierten Charakterisierung der hergestellten Nanostrukturen, beinhaltet die Arbeit auch neue Ansätze für die Formulierung intelligenter Nanostrukturen und belegt deren Eignung für die Verkapselung und Freisetzung pharmazeutischer Wirkstoffe.

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List of abbreviations

AA	Acrylic acid
AHPC	6-Acryloyloxyhexylpyridinium chloride
AIBN	Azodiisobutyronitrile
BAC	*N,N'*-Bis(acryloyl)cystamine
BIS	*N,N'*-Methylene bisacrylamide
CLSM	Confocal laser scanning microscopy
Con A	Concanavalin A
Cryo-TEM	Cryo-transmission electron microscopy
CTA	Chain transfer agent
Đ	Dispersity
DLS	Dynamic light scattering
DMDOMA	*N*-[(2,2-Dimethyl-1,3-dioxolane)methyl]acrylamide
DMF	Dimethylformamide
DOX	Doxorubicin
DP	Degree of polymerization
DTT	D,L-Dithiothreitol
EPR	Enhanced permeability and retention
ITC	Isothermal titration calorimetry
KTDA	Ketal containing diacrylate
LCST	Lower critical solution temperature
M_n	Number average molar mass
NG	Nanogel
NiPAm	*N*-Isopropylacrylamide
NMP	Nuclear magnetic resonance
OEGA	Oligo(ethylene glycol) acrylate
PAM	Poly(*N*-acryloyl morpholine)
PAMP	Poly(*N*-acryloyl-*N*'-methyl piperazine)
PCL	Polycaprolactone
PDI	Polydispersity index
RAFT	Reversible addition-fragmentation chain transfer
SEC	Size exclusion chromatography
SEM	Scanning electron microscopy
TCEP	Tris(2-carboxyethyl)phosphine
TEM	Transmission electron microscopy
VCL	*N*-Vinylcaprolactam

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2015 - present	PhD studies in Polymer Chemistry, Friedrich Schiller University Jena (Prof. Ulrich S. Schubert), Germany Topic: " Design of responsive and degradable supramolecular host-guest polymer nanostructures for therapeutic applications"
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Declaration of authorship / Selbständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig angefertigt, nicht anderweitig zu Prüfungszwecken vorgelegt und keine anderen als die angegebenen Hilfsmittel verwendet habe. Sämtliche wissentlich verwendete Textausschnitte, Zitate oder Inhalte anderer Verfasser wurden ausdrücklich als solche gekennzeichnet.

I hereby certify that the work disclosed here is, to the best of my knowledge, original and the result of my own investigations, except as acknowledged, and has not been submitted, either in part or whole, for a degree at this or any other university.

Jena, den _____

Publications P1 to P5